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A paper-based chemiluminescence immunoassay device for rapid and high-throughput detection of allergen-specific IgE†

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The fast and precise detection of potential allergen-specific immunoglobulin E (sIgE) is imperative for the diagnosis and appropriate treatment of allergic diseases. In this study, we have successfully fabricated a novel paper-based immunoassay device for the detection of sIgE in allergic diseases. We used Can f 1, one of the main dog allergens, as a model allergen to detect sIgE in human sera. To achieve excellent performance, the experimental parameters were optimized. Further, we extended this device for potential applications in the clinical diagnosis of allergic diseases: worthwhile clinical performance in the detection of allergens was achieved as compared to that achieved by commercial enzyme-linked immunosorbent assay (ELISA) kit. Therefore, it was proven that this strategy has the advantages of high-throughput, rapid, sensitive, and highly accurate detection of trace amounts of sIgEs. Furthermore, by simply changing the antigen and antibody, this device could be used for the high-throughput detection of other allergens, so as to achieve multiallergen detection and appropriate desensitization therapy, thereby making it promising in the determination of allergic diseases in clinics.

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Introduction

During the last five decades, the worldwide prevalence of allergic diseases has grown dramatically, and they have become one of the six major ailments in the world.^{1–3} Actually, more than 25% people suffer from various allergic diseases, such as hay fever, food allergies, atopic dermatitis, and allergic asthma.^{4–7} Many allergic patients suffer from sneezing, coughing, itching, swelling, sinus pain, and shortness of breath, which is considerably painful to them, representing a major health problem in modern society.⁸

The occurrence of allergic diseases is directly related to allergen-specific immunoglobulin E (sIgE),^{9–11} which could be produced by B cells when allergens enter the body for the first time.¹² After being expressed, sIgE binds to IgE high-affinity

receptors (FcεRI) on the mast cells and basophils, leaving the host body in a sensitizing phase. The next encounter of the allergens leads to its recognition with the FcεRI-associated sIgEs, which causes the aggregation of FcεRI receptors and activation of basophils or mast cells to release certain inflammatory factors, triggering allergic symptoms.¹³ Keeping away from allergens has been considered to be one of the most important methods in the radical cure of allergic diseases.¹⁴ Therefore, the fast and precise determination of the potential allergen sIgE is urgently necessary for the diagnosis and appropriate treatments of allergic diseases.

Skin-prick testing (SPT) is the most common diagnostic testing method in clinics.^{15,16} Allergens are delivered to the patients by pricking the skin with needles. It is imperative that the suspected allergen sources are nontoxic and cannot simultaneously screen reactivity to many different allergens.¹⁷ Moreover, SPT can induce pain and discomfort to the patients and is unsuitable for patients with severe symptoms and cannot stop taking medications.^{18,19} For these reasons, a variety of *in vitro* serological tests to detect serum sIgEs have been developed in clinical practice, including radio allegro sorbent test (RAST), fluorescence enzyme assays (CAP-FEIA, immunoCAP), and enzyme-linked immunosorbent assay (ELISA). Due to its inefficiency and high cost, the once market-leading RAST method is no longer popular.^{20,21} This assay has been replaced by the CAP-FEIA technique, which can provide a

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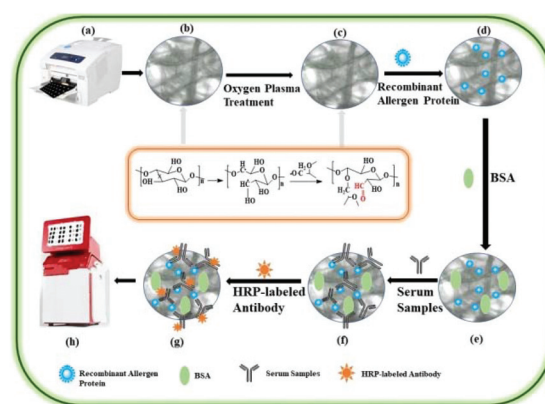
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quality-controlled and accurate assay for the *in vitro* detection of sIgEs.^{22,23} However, CAP-FEIA is based on a singleplex assay format and requires an individual assay for each allergen, increasing costs and burden on the patient.^{24–26} Therefore, CAP-FEIA is not suitable for first-line screening because of the restricted number of allergens in the process of simultaneous detection. Recently, ELISA has been widely used as a sensitive and selective method for sIgE measurement, in which sIgEs could be spectrophotometrically detected by measuring the absorbance of the assay solution.²⁷ However, ELISA often consumes large volumes of analytes and requires long incubation time for the entire process.²⁸ Therefore, an alternative method that could overcome the above main drawbacks, including the requirement of costly, time-consuming, large quantities of serum samples and/or special laboratory environments with sophisticated equipment and trained personnel, has become imperative. Meanwhile, the currently available serological tests primarily rely on crude allergen extracts, which are prepared from various allergen-containing biological materials (e.g., foods, pollens, *etc.*).²⁹ However, these extracts contain a variety of allergenic and nonallergenic components and it is often difficult to standardize their allergen content or potency. To resolve this issue, recombinant technology as the basis of component-resolved diagnosis of allergens has been introduced into clinical routines to improve the diagnosis accuracy of each individual patient's risk.^{30,31}

Cellulose paper, a common commodity in daily life, was endowed with extremely important functionality when George Whitesides and co-workers fabricated a paper-based analytical device for point-of-care (POC) diagnostics in 2007.^{32–34} Due to their distinct merits such as good biocompatibility, high efficiency, low cost, and portability, paper-based devices have been used in numerous applications.^{35–38} Further, it has been widely investigated as an emerging analytical method for the detection of biologically interesting molecules by converting and amplifying a biomolecular recognition event to a dateable semiquantitative or quantitative signal.^{39–42} Till date, a variety of paper-based sensors for the fast and efficient detection of target molecules have been developed.^{43–48} Paper-based sensors are affordable in resource-constrained environments when compared with advanced diagnostic techniques.^{49–51} Among them, the paper-based ELISA (P-ELISA) system is noteworthy as it offers a simple, but universal, platform for the detection of antibody–antigen interactions. These immunoassays, often fabricated by antibodies immobilized on filter paper, have proven to be quick, simple, and cost-effective alternatives to conventional ELISA. Usually, these prepared immunoassays are most often combined with colorimetric detection for onsite test results.⁵² Other means of detection have also been reported, such as electrochemical detection and chemiluminescence (CL).^{53,54} The development of paper-based immunoassays and other microfluidics related to POC diagnostics have the potential to simplify and accelerate the current workflow of setting a diagnosis.^{55,56} For example, Khan *et al.* developed instantaneous blood-typing tests using specific antibody–antigen interactions on paper-based analytical

devices.⁵⁷ Zhao *et al.* introduced a paper-based immunodevice, which was successfully performed for carcinoembryonic antigen (CEA) detection in human serum and the detection limit was 30 times lower than the clinical CEA level.⁵⁸ Hsu *et al.* formulated a P-ELISA system to detect NC16A auto-immune antibodies.⁵⁹ This system could diagnose and monitor bullous pemphigoid activity. When compared to conventional ELISA, P-ELISA has proven to be faster and less costly.⁶⁰ Moreover, it conveys a similar level of sensitivity and specificity while using tiny amounts of clinical samples.^{61–63} Paper-based materials have achieved worthwhile achievements in certain fields; however, to the best of our knowledge, they have never been involved in allergic diseases. Fundamentally, allergic diseases are caused by antigen–antibody binding, and P-ELISA can be a very promising diagnostic method to overcome the shortcomings of traditional methods such as RAST, CAP-FEIA, and ELISA.

Consequently, herein, we reported a novel paper-based chip for detecting sIgEs. In this study, we used Can f 1, one of the main dog allergens, as the model allergen to assess allergic diseases.⁶⁴ As shown in Scheme 1, after the modification of aldehyde groups with oxygen plasma treatment, recombinant Can f 1 was subsequently immobilized on the surface of the paper to fabricate a paper-based chip. Thereafter, the steps of traditional ELISA were followed, including the subsequent incubation with bovine serum albumin (BSA), sIgEs, and horseradish peroxidase (HRP)-labeled antibody (anti-IgE). The detection of sIgE to Can f 1 in human serum was eventually achieved by CL immune assay. Currently, CL is a common method for paper-based detection. As shown in Scheme S1,† the antibody labeled by HRP usually uses luminol as the substrate. When hydrogen peroxide exists, luminol undergoes an oxidation reaction to form intermediates at excited states. As



Scheme 1 Schematic illustration of paper-based immunoassay. (a–b) Fabrication of wax microzones on paper; (c) structure of paper zone with aldehyde group modification; (d) recombinant allergen protein was immobilized on paper zone through Schiff base linkage; (e) free aldehyde groups were blocked by BSA; (f) sIgEs in the serum samples bonded with the allergen; (g) final label of HRP-labeled antibody (anti-IgE) immune-reacted with sIgEs; and (h) CL signal response from the CL analyzer.

they return to the ground state, the emitted luminescence signals are measured by the instrument for analysis. Generally, this novel system provides the following key features: (1) simple and inexpensive method for fabricating paper-based devices; (2) fast determination of sIgE levels with high accuracy; and (3) programmable microzones and independent detection units on the paper, thereby achieving high-throughput allergen screening. Furthermore, the critical parameters of paper-based devices were optimized, and the analytical performance was determined. Finally, we also extended it to potential applications in the clinical diagnosis of allergic diseases, and worthwhile clinical performance in the detection of allergens was achieved by contrasting its performance with that of commercial ELISA kits.

Experimental

Materials and reagents

BSA was purchased from BioFroxx. Phosphate buffer solution (PBS) (10 mM, pH = 7.4) was used as a coupling buffer for antibody immobilization. PBS containing 2% BSA was used as a blocking buffer for the residual reactive sites. Whatman chromatography paper #1 (WCP#1) (200 mm × 200 mm) was purchased from Shanghai Si Rui Technology Co., Ltd. Schiff reagent was purchased from Nanjing Best Biological Technology Co., Ltd. Anti-IgE and HRP-labeled signal goat anti mouse IgG (anti-IgG) were obtained from Kirkegaard & Perry Laboratories, Inc. and isopropyl-*b*-D-thiogalactopyranoside (IPTG) was purchased from TakaRa Biotechnology (Dalian) Co., Ltd. A 96-well microplate, 3,3',5,5'-tetramethylbenzidine (TMB) color liquid, and nickel column were purchased from Costar (USA), Nanjing KeyGen Biotech. Co., Ltd. The sera from 42 dog-allergy patients with allergic symptoms and positive SPT results (allergens were supplied by ALK-Abello, Inc., Horsholm, Denmark) and 23 negative sera from healthy subjects without a history of allergic symptoms (normal human sera, NHS) were provided by the First Affiliated Hospital of Nanjing Medical University. All the negative sera were clinically tested true-negative samples. These sera were further confirmed by ImmunoCAP (Thermo Fisher, Fremont CA). Further, 10 out of the 42 dog-allergy patients' sera and 5 out of the 23 negative sera were used to optimize the incubation time for serum samples. Then, 50 out of the 65 samples were randomly selected to verify the applicability of paper-based devices. The study protocol was approved by the ethical committee of the First Affiliated Hospital of Nanjing Medical University. Written informed consent for the use of blood samples were obtained from all the participants before study entry according to the Declaration of Helsinki. All the chemicals and reagents were of the analytical grade and used without any further purification.

Ethical statement

All the experiments were performed in accordance with the Guidelines (No. 20170201) and approved by the ethics commit-

tee at Nanjing Tech University (Nanjing, China). Informed consents were obtained from human participants in this study.

Measurements and instruments

The paper-based chip was created by ColorQube 8580N wax printer (Xerox, Japan). The aldehyde-group-modified paper-based chip was fabricated by means of oxygen plasma treatment with the Schwarze P30C device (Beijing Jiarun Power Technology Co. Ltd, China). The chemical modification process was confirmed through the FT-IR spectra by using a Nicolet IS50 instrument (Thermo Fisher, USA). The scanning electron microscopy (SEM) images were recorded on a JSM-7800F scanning electron microscope (JEOL, Japan). The CL intensity was measured and recorded with the Omega Lum W CL analyzer (Aplegen, USA).

Recombinant Can f 1 expression and purification

Can f 1 is a dog allergen found in dog dander and saliva. It has 525 bases pairs and encoded 174 amino acids. The Can f 1 gene was optimized on the basis of the unchanged amino acid sequences, according to the GenScript rare codon analysis tool (http://www.genscript.com/cgi-bin/tools/rare_codon_analysis), and synthesized (GenScript, Nanjing). It was then subcloned into the pET-28a vector with the sites of EcoR I and Xho I and verified by DNA sequencing. The pET28a-Can f 1 plasmid was then transformed into BL21 (DE3) pLysS *E. coli*. A positive colony was selected and incubated at 37 °C overnight. The entire broth was inoculated into 300 mL LB-kanamycin broth and incubated at 37 °C under shaking at 200 rpm until the optical density (OD) value at 600 nm reached 0.6–0.8. IPTG was added to the broth with the final concentration of 1.0 mM and incubated for another 6 h. The bacterial cells were gathered by centrifugation at the speed of 3600g at 4 °C for 30 min, and the sediment was harvested. The sediment was lysed in a lysis buffer by sonication at 20 kHz with 5 s pulse-on and 5 s pulse-off. After collection by centrifugation at 12 000g at 4 °C for 30 min, the supernatant was loaded on the nickel column, washed with running buffer containing 500 mM NaCl, 50 mM NaH₂PO₄, 10 mM imidazole, pH 8.0, and eluted in order with elution buffer containing 500 mM NaCl, 50 mM NaH₂PO₄, and 30, 50, 100, 150, 200, and 250 mM imidazole, at pH 8.0; all the eluted fractions were collected. The purified protein was then placed in a dialysis bag. The dialysis bag was sealed and put into a PBS solution for 32 h; the PBS solution was changed every 8 h.

Immunoreactivity of human sera with recombinant Can f 1 by ELISA

Here, 10 real serum samples were used to confirm the immunoreactivity of human sera with recombinant Can f 1 by a conventional ELISA kit. The 96-well microplate was coated with 2 µg per 100 µL per well (0.02 µg µL⁻¹) of recombinant Can f 1 in PBS overnight at 4 °C. After being washed with PBS-T (PBS containing 0.1% Tween 20) (200 µL per well) for three times, the plates were then blocked with BSA (1 mg mL⁻¹, 200 µL per well) for 1 h at 25 °C and washed with

PBS-T. After that, the plates were incubated with 100 μL of the patients' sera (1 : 100 v/v in PBS) at 25 $^{\circ}\text{C}$ for 2 h and washed with PBS-T for three times. Next, the plates were incubated with 1 : 4000 v/v diluted anti-IgE at 25 $^{\circ}\text{C}$ for 1 h and washed with PBS-T for three times, followed by the addition of 100 μL TMB color liquid to each well. 30 min after coloration, the reaction was stopped by adding 50 μL of 3 M H_2SO_4 . After incubation, the plates were processed as described and the absorbance was read at 450 nm (Biotech Eon, Biotech, USA). Here, 5 out of the 23 NHS were used as control. If the OD value of the sample was 2.1 times higher than the mean OD value of NHS, the result was considered positive; otherwise, it was considered negative.

Fabrication of paper microzone plates

A microzone plate with a diameter of 3 mm was prepared according to a previously reported method with slight modification.⁶⁵ Firstly, the paper was treated with a plasma cleaner for 30 min to produce numerous aldehyde groups on the surface of the paper. Then, the paper microzone plates were formed by the wax printing technique, in which a designed program was prepared by using drawing software and printed onto paper using a wax printer. Next, in order to form complete hydrophobic barriers among the formed microzones on paper, the printed paper was kept in an oven and baked at 100 $^{\circ}\text{C}$ for 60 s. Finally, the wax-penetrated paper was obtained after cooling to room temperature. The modified process was successfully confirmed by SEM and FT-IR measurements.

Validation of experimental route with model protein

We used β -actin as a model protein to verify the feasibility of protein immobilization on a paper-based device. First, 3 μL of cell lysate was added on the paper and incubated for 30 min. After that, the microzones were washed three times with PBS (10 μL). Next, 5 μL BSA was applied to the microzones for 30 min to block the aldehyde groups. After washing for three times with PBS, 3 μL β -actin antibody and 3 μL anti-IgG were successively dropped onto the paper. Each step was incubated for 30 min and washed with PBS for three times to wash away the unbound substances. Finally, 5 μL BeyoECL Plus was dropped onto the paper zones to produce a CL signal.

Optimization of assay conditions on paper-based chips

The experimental parameters were optimized by changing the assay conditions, including the concentrations of BSA, antigen, and antibody. At the same time, the incubation time of the antibody was also optimized. First, to optimize the amount of BSA, different concentrations of BSA from 0 to 5% were added to observe the change in CL intensities. Meanwhile, the concentration of antigen was maintained at 20 $\mu\text{g mL}^{-1}$ and anti-IgE at a dilution level of 1/1000. Similarly, to optimize the antigen concentration, it was changed from 4 to 100 $\mu\text{g mL}^{-1}$ with BSA concentration of 2% and anti-IgE concentration of 1/1000 dilution. Meanwhile, we also optimized the range of anti-IgE concentration from 1/500 to 1/2000 under the condition of 20 $\mu\text{g mL}^{-1}$ antigen and 2% BSA.

The incubation time of the antibody was finally optimized under the optimum parameters obtained by screening to obtain the optimal incubation time for distinguishing positive serum samples. By optimizing these parameters, we obtained the best combination of parameters.

Procedure for sIgE detection with paper-based devices

The 10 abovementioned samples of real sera were also used to verify the feasibility of paper-based devices under optimized conditions. The recombinant antigen, serum sample, anti-IgE, and exposure solution were added to the paper microzones in order to obtain the CL signal. Firstly, 3 μL of the 0.17 mg mL^{-1} recombinant allergen protein was fixed on the paper *via* Schiff base linkage. Then, the paper microzones were washed three times by using 10 μL of PBS each time. Secondly, 3 μL blocking buffer was applied to each paper zone and incubated for 30 min at room temperature. Then, the paper zones were washed with PBS for three times. Thirdly, for the covalent immobilization of targeted sIgE protein, 3 μL human serum sample solutions were applied to each paper zone. After incubating for 15 min, each paper zone was thoroughly washed with PBS three times to wash away the excess proteins. Then, 3 μL of 1/1000 anti-IgE was added to each paper zone and incubated for 10 min. The detection zone was then ready for subsequent CL assay after washing. At last, 5 μL BeyoECL Plus was dropped onto the paper zones to produce a CL signal. As shown in Scheme 1, the CL signal was measured by a computerized luminescence analyzer. The paper-based device was put on the bottom of the machine holder that was fixed on the bottom of the sample plate. When the CL intensity was detected, it would take photographs. The sample concentration could be quantified by the brightness of the CL signal. Here, 5 out of the 32 NHS were used as the control. If the CL intensity of the sample is 1.6 times higher than the mean CL intensity of NHS, the result is considered positive; otherwise, it is considered negative.

Comparison of traditional ELISA and p-ELISA in double-blind experiments

We introduced 50 human serum samples to detect the allergen reaction for evaluating the applicability of the paper-based device. The Can f 1 sIgEs in 50 different serum samples were simultaneously determined by traditional ELISA and our paper-based device in a randomized, double-blind manner. The samples comprised both dog-allergy patients' sera and NHS. Their serial numbers were randomly assigned and unknown to the experimenters. Then, we collected and analyzed these data from the two methods.

Results and discussion

Expression and purification of Can f 1

The codon optimization of the Can f 1 sequence was subcloned into pET-28a (+) vector and transformed into BL21 (DE3) pLysS *E. coli*. A comparison of the sequences before and

after optimization is shown in Fig. S2a†. By sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), it was discovered that Can f 1 was mainly expressed in the supernatant (Fig. S2b†). Then, the Can f 1 recombinant was purified by a Ni column. The purified Can f 1 was visible as a single band as identified in the SDS-PAGE data. The apparent molecular weight of purified Can f 1 was 19 kDa (Fig. S2b†).

Immunoreactivity of human sera with recombinant Can f 1 by ELISA

The ability of Can f 1 to bind sIgEs in the dog-allergy patients' sera were detected by a commercial ELISA kit. The patients' sera samples included 6 numbers of dog-allergy positive sera and 4 negative controls. As shown in Table S1 and Fig. S3,† 6 out of the 10 dog-allergy patients' sera showed positive IgE reactivity to Can f 1 and 4 samples were negative. Then, we used these samples with known results to verify our paper-based device.

Fabrication and characterization of paper-based devices

The design of our paper-based device for allergen reaction detection is illustrated in Scheme 1. This paper-based device was printed by wax printing, which is low cost, simple, and rapid. After one step of plasma treatment, the hydroxyl group on the carbon radical could lose a hydrogen atom, resulting in the formation of an oxygen radical. Then, the single electron on this oxygen radical combined with an electron in carbon, leading to the generation of an aldehyde group. In this way, the aldehyde group was chemically modified on the paper surface; therefore, the antibody/antigen could be directly immobilized on the paper through the reaction between the aldehyde and amino groups. Obviously, the production of the aldehyde group on the fiber structure was necessary in the entire immobilization procedure. The FT-IR spectra were obtained to confirm this modification of the aldehyde group. As shown in Fig. S4,† the absorption peak at 1640 cm^{-1} , which corresponds to the carbonyl in the aldehyde group, is obviously enhanced when compared with that without plasma treatment. Further, when the antibody was immobilized on paper, the amino group of the antibodies was covalently coupled with the aldehyde group to form a Schiff base.⁶⁶ The absorption at 1640 cm^{-1} was significantly decreased due to the reducing of the aldehyde group. In addition, we also used a Schiff reagent to confirm the existence of aldehyde groups on the paper surface. A Schiff reagent, also known as a magenta aldehyde reagent, can react with aldehyde groups and turn into a purple red solution. As shown in Fig. S5,† the paper gradually turned from white to purple red, which was visible with the naked eyes, when the Schiff reagent was dropped onto the microzones of the paper, indicating the existence of aldehyde groups.

In order to further monitor the modification process, the surface morphologies of the paper before and after the plasma treatment were investigated by SEM to verify the feasibility of the immobilized antigen, which is shown in Fig. S6.† Before oxygen plasma treatment, the structure of every bundle fiber

was smooth (Fig. S6a†). However, the surface morphology of the paper became rough with small stings and cracks appearing on the fiber structure upon plasma treatment (Fig. S6b†). These changes could increase the specific surface area, which is beneficial for antibody immobilization. Interestingly, the roughness of the paper surface considerably decreased after the antigen or BSA were immobilized on the paper (Fig. S6c and S6d†).

Verification of mechanism for Can f 1 sIgEs detection

Herein, the proposed mechanism for the detection of Can f 1 sIgEs is the P-ELISA system. First, the aldehyde group on the paper surface reacted with the amino group of Can f 1 to form a reversible Schiff base. After the Can f 1 was successfully captured on the microzone plate, BSA was added to block the residual aldehyde groups. Next, we incubated the patient's serum, in which the sIgEs could specifically bind with Can f 1. Anti-IgE was introduced as the secondary antibody to interact with sIgEs. Therefore, the detection of sIgEs in human serum was eventually achieved by CL immunoassay.

In order to verify the feasibility of the CL reaction, we first used β -actin as the model to detect the CL signals in our paper-based device. The cell lysate containing β -actin was added on the microzone plate and incubated for 30 min. After washing with PBS, BSA was applied to block the aldehyde groups, which could avoid the undesired capture of antibodies. Next, we successively incubated the microzone plate with β -actin antibody and anti-IgG. As shown in Fig. 1a and b, the CL images of β -actin (column 2) showed significant signals after the BeyoECL Plus was dropped onto the paper zone as comparison to the treatment only with BSA (column 1). However, without BSA blocking, significant CL signals could be observed regardless of the fact that β -actin was added (column 3) or not (column 4). Therefore, to eliminate interference from false positives, it is necessary to add BSA to block

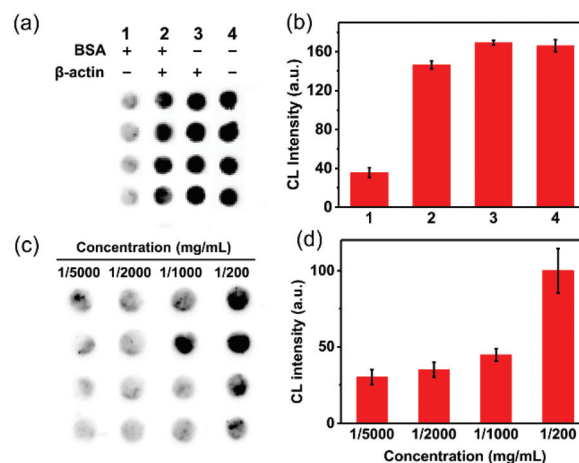


Fig. 1 (a) CL images of the β -actin combined on paper and (b) their corresponding CL intensities; (c) CL images of microzone with different concentrations of β -actin and (d) their corresponding CL intensities. All the experiments were conducted in quadruplicate.

the reactive groups on the paper. Furthermore, the CL intensities gradually enhanced with further additions of β -actin, indicating that the sensitive determination of β -actin with this paper device could be achieved (Fig. 1c and d). Therefore, these results demonstrated that this device could specifically respond to the target protein through a rationally designed procedure.

Optimization of experimental conditions for serum samples

In order to enhance the sensitivity of paper-based chips in the detection of allergen-specific IgE, the experimental conditions were comprehensively studied. The incubation time with the real sample in the ELISA system is also a key factor that affects the time efficiency of the method. Therefore, the effect of incubation time for CL immunoassay in the paper immune-plate was examined at room temperature. The CL intensities of the four serum samples were recorded for two different incubation times (15 min and 30 min). It is mentioned that samples 1 and 2 were positive serum samples, while 3 and 4 were negative serum samples. As shown in Fig. 2a, significant differences in the CL signals between the positive and negative samples could be observed for 15 min incubation. In addition, the overall CL intensities were increased with incubation time increased to 30 min between the antigen sample and captured antibodies (Fig. 2b and c). Notably, the CL intensities of the positive serum samples (samples 1 and 2) with 30 min incubation were about 1.1 times higher than those with 15 min incubation. However, the CL intensities of the negative samples (samples 3 and 4) incubated for 30 min increased over 2 times than those with 15 min incubation. This led to a lower discrimination of the negative and positive serum samples after the increase in incubation time. Therefore, considering the optimal analytical performance and actual operation, the incubation time of 15 min for the captured anti-

bodies and antigen sample's immunoreaction was selected for the following study.

Optimization of concentrations of antigen, BSA, and second antibody

The concentrations of antigen, BSA, and second antibody are very important for improving the detection accuracy. We used the positive sample that contained the targeted protein (sIgE) to determine these parameters. The CL intensity profiles were recorded by changing the concentrations of antigen, BSA, and second antibody. First, we optimized the antigen concentration, which could be covalently immobilized on the plasma-treated paper, and subsequently, it combined with sIgE and HRP-labeled second antibody to affect the CL intensity. As shown in Fig. 3a and b, the signals in the CL images gradually enhanced with the antigen concentration increasing from $4 \mu\text{g mL}^{-1}$ (1/50) to $100 \mu\text{g mL}^{-1}$ (1/2). Notably, the CL intensities calculated by image J reached the maximum at the concentration of $20 \mu\text{g mL}^{-1}$ (1/10). Therefore, antigen concentration of $20 \mu\text{g mL}^{-1}$ was selected in this study.

Similarly, we then optimized the BSA concentration by recording the CL intensity changes in the presence of different BSA amounts ranging from 0 to 5%. As shown in Fig. 3c and d, the CL intensity was extremely high in the absence of BSA (concentration = 0%), which is due to the failure of the antigen to occupy all the reaction sites of the aldehyde groups on the paper; therefore, the second antibody could combine with the non-occupied sites to produce CL signals. With the

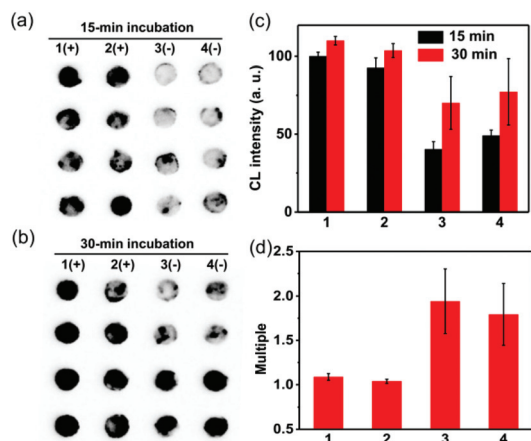


Fig. 2 CL images of 4 serum samples incubated with the paper device for (a) 15 min and (b) 30 min; (c) corresponding CL intensities of (a) and (b); (d) increased folds of different serum samples incubated with the paper device from 15 to 30 min. All the experiments were conducted in quadruplicate.

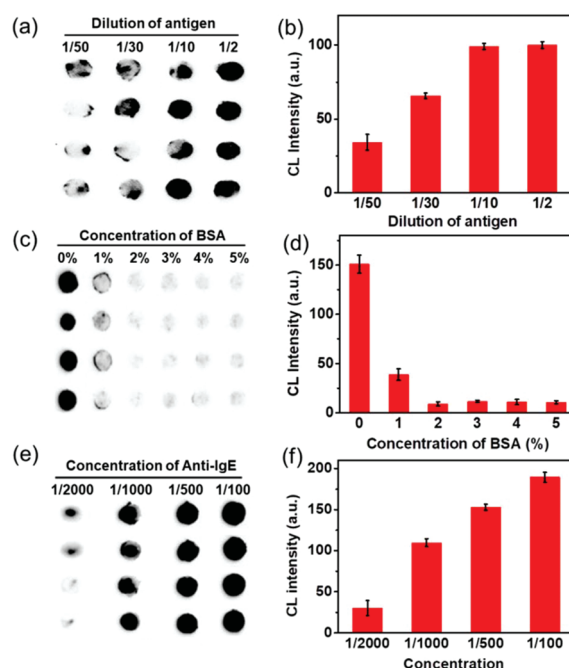


Fig. 3 (a) CL images of different concentrations of antigen and (b) their corresponding CL intensities; (c) CL images of different concentrations of BSA and (d) their corresponding CL intensities; (e) CL images of different concentrations of anti-IgE and (f) their corresponding CL intensities. All the experiments were conducted in quadruplicate.

increase in the BSA concentration, an increased number of residual aldehyde groups are blocked, resulting in a continuous decrease in the CL intensity. Obviously, the CL intensity reached its lowest level at the BSA concentration of 2%, indicating the reactive sites of aldehyde groups were almost blocked. In addition, due to the large molecular weight of BSA, it might cover the antigen and interfere with subsequent immune responses. Furthermore, the viscosity of the BSA solution at higher concentrations would increase the difficulty to wash away the residual proteins. Taking the abovementioned facts into consideration, the concentration of BSA at 2% was selected as the best choice.

In order to decrease the analysis cost, one of the most effective ways is to reduce the volume of the second antibody solutions into the paper zone. Therefore, the optimization of the second antibody amount was performed at an antigen concentration of $20 \mu\text{g mL}^{-1}$ and BSA concentration of 2%. As shown in Fig. 3e and f, the CL intensity increased in proportion to the second antibody concentration from 1/2000 to 1/100. It should be noted that the higher concentration of the second antibody leads to excessive CL intensity, which might decrease the discrimination between the negative and positive serum samples. In addition, the analytical cost increases along with the increase in the second antibody amount. The CL intensity indicated that uniformity and sufficiency of the CL signal in the paper zone could be achieved at 1/1000 dilution. Thus, the second antibody concentration of 1/1000 was selected as the optimal condition for subsequent human serum sample analyses.

Effects of pH and temperature on paper-based devices

Both pH and temperature have an effect on paper-based devices with respect to the detection of allergen-specific IgE, which has been investigated. As shown in Fig. S7,† the CL intensity of paper-based devices first increased and then decreased as pH ranged from 4.0 to 10.0: the maximum CL intensity was achieved at pH 7.4. Furthermore, the incubation temperature was also an important parameter affecting the analytical performance of the paper immune-plate. To obtain optimal CL signals, the effect of temperature was investigated. As shown in Fig. S8,† from 4 to 34 °C, the CL signal increased, while it decreased as the incubation temperature increased from 37 to 44 °C. However, there was a marginal difference in the CL intensity between 24 and 37 °C. Considering the savings in cost and number of steps, 24 °C could be used as the optimal incubation temperature to detect samples because it is also the room temperature in a laboratory. Therefore, room temperature was selected as the incubation temperature for subsequent experiments.

Evaluation of reproducibility and stability of paper-based detection

After establishing the optimal conditions, we subsequently used them to detect the above 10 serum samples, which were identified with ELISA. As shown in Fig. 4, 6 out of the 10 dog-allergy patients' sera showed positive IgE reactivity to Can f 1;

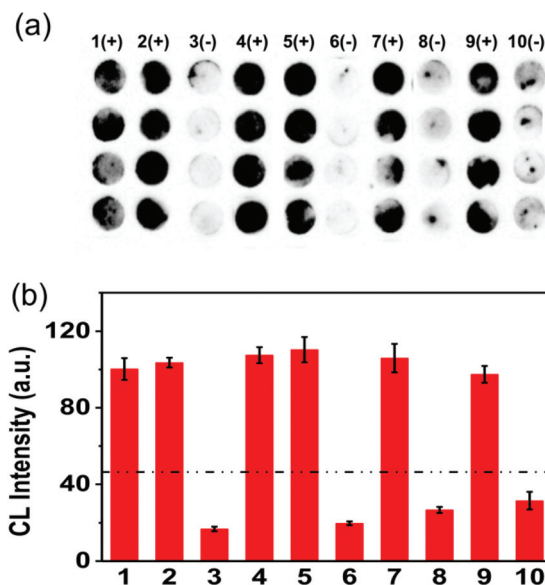


Fig. 4 (a) CL images of 10 serum samples determined by the paper-based device and (b) their corresponding CL intensities. Dotted line indicates 1.6 times the mean CL intensity of 5 NHS. All the experiments were conducted in quadruplicate.

4 negative samples were also confirmed. This is consistent with the results obtained with the commercial ELISA kit (Fig. S3†). Therefore, this experiment further confirmed the feasibility of paper-based devices for the detection of allergic reactions in real samples.

In addition, reproducibility and stability were major concerns for practical applications of paper-based devices. The reproducibility of the as-prepared paper-based device was investigated by different experimenters under the same conditions. Further, the relative standard deviation (RSD) values were below 6.6% for the same concentration of serum samples. Therefore, operational errors of different experimenters in actual serum samples are not obvious. With regard to the evaluation of stability of paper-based devices for the detection of serum samples, we stored the paper-based device at 4 °C. As shown in Fig. 5, when the CL intensity of the negative serum samples was modified to the same level, it could be found that the CL intensity of the positive serum samples slightly decreased with an increase in storage time. When the paper-based device was preserved at 4 °C over 30 days, the discrimination between the positive and negative serum samples decreased, but it was still distinguishable. In short, the paper-based device had good stability and reproducibility.

Applicability of paper-based devices with respect to traditional ELISA in real samples

The applicability of our paper-based device was validated by a randomized, double-blind trial. The 50 different serum samples were determined by both traditional ELISA and our paper-based device. The samples were composed of Can f 1 sIgE patients' sera, other dog allergy sera, and NHS samples, numbers of which were randomly assigned and unknown to

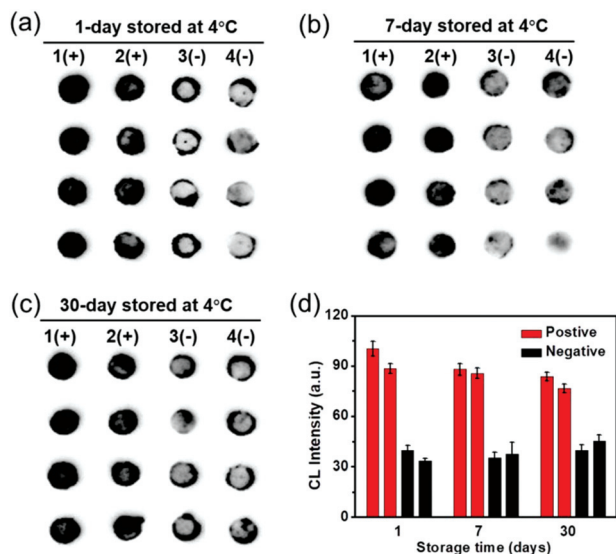


Fig. 5 CL images of 4 serum samples (samples 1 and 2 are positive (+); samples 3 and 4 are negative (-)) detected by the paper device with different storage times for (a) 1 day, (b) 7 days, and (c) 30 days. (d) Corresponding CL intensities of (a–c). All the experiments were conducted in quadruplicate.

the experimenters. From the scatter plot shown in Fig. 6, it is evident that the detection results of paper-based devices and ELISA kit show a preferable correlation distribution. Further, as shown in Fig. S9 and Table S2,† in comparison with traditional ELISA, there is no significant difference in the two samples; a 4% error rate was determined between the results measured by the paper-based immunoassay device and ELISA results. Furthermore, we verified the accuracy for each micro-zone in each sample; all of them were far beyond 60% (Table S3†). In fact, ELISA kit analysis is a widely used analytical technique in molecular biology to detect allergic reactions in clinics. However, this technique is often time-consuming and

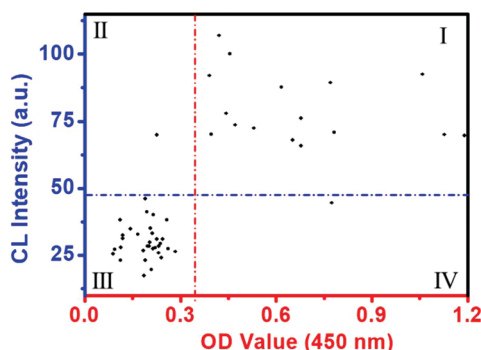


Fig. 6 Scatter plot of correlation distribution relationship between p-ELISA and ELISA kit. X axis for the OD values at 450 nm of 50 serum samples determined by ELISA kit and Y axis for the CL intensity of 50 serum samples determined by the paper-based device. Red dotted line indicates 2.1 times the mean OD value of 5 NHS. Blue dotted line indicates 1.6 times the mean CL intensity of 5 NHS.

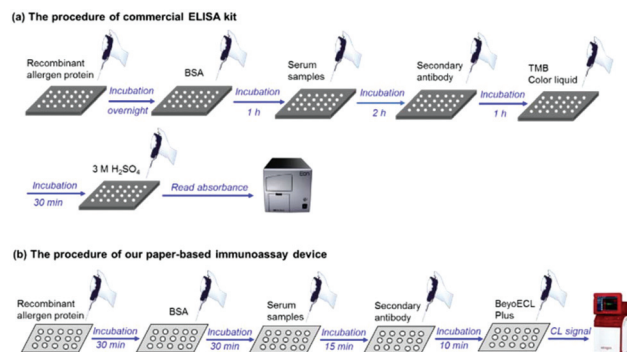


Fig. 7 Schematic representation of the test procedures for (a) commercial ELISA kit and (b) paper-based immunoassay device.

incurs higher costs. As shown in Fig. 7, our method could dramatically reduce costs and save time as compared to conventional ELISA analysis. Therefore, this paper-based device could provide a simple, time-saving, low-cost, and highly accurate method that would hold promising potential as a feasible alternative method for the determination of sIgE in real samples.

Conclusions

In summary, we have successfully fabricated a paper-based immunoassay device and proved its feasibility for the accurate detection of Can f 1 sIgEs. Under the oxygen plasma treatment for several minutes, this paper-based immunodevice could be quickly constructed with aldehyde group modification, which subsequently reacted with the amino group of Can f 1 to detect the allergen (sIgE). The covalent immobilization of antigen through a Schiff base as the linkage could enhance the stability of antibodies on the paper. Therefore, the paper-based device could be used for the precise detection of the allergen (sIgE). In addition, combining the cost-effectiveness of paper-based devices and the sensitivity of CL-ELISA, we have proven that this strategy has the advantages of the high-throughput, rapid, sensitive, and highly accurate detection of trace amount of sIgEs in real biological samples. Furthermore, by simply changing the antigen and antibody, this device can be used for the high-throughput detection of other allergens, so as to achieve multiallergen detection and appropriate desensitization therapy, thereby showing promising potential in the determination of allergic diseases in clinic.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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Supporting Information

A paper-based chemiluminescence immunoassay device for rapid and high-throughput detection of allergen-specific IgE

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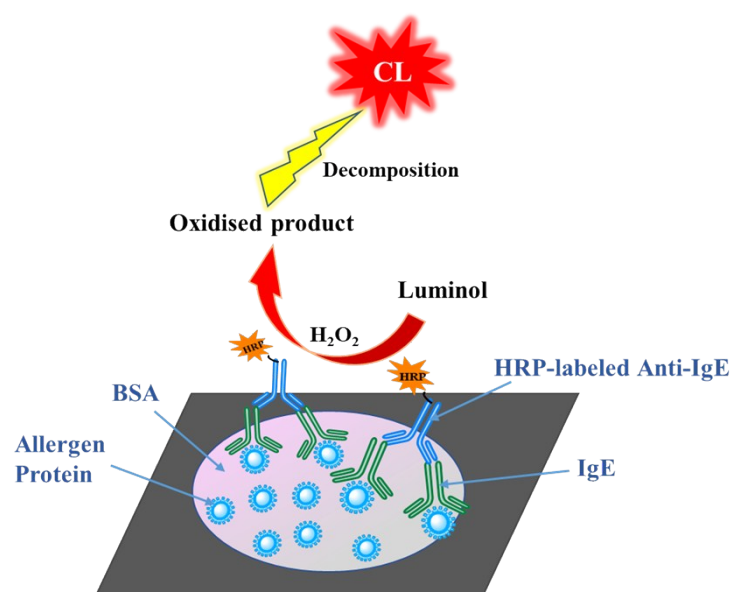


Fig. S1. The illustration of chemiluminescence mechanism in this assay.

(a)

	1	60
Before	ATGAAGACCCTGCTCCTCACCATCGGCTTCAGCCTCATTGCGATCCTGCAGGCCAGGAT	
After		CAGGAC
		Q D
	61	120
Before	ACCCAGCCTTGGGAAAGGACACTGTGGCTGTGTCAGGGAAATGGTATCTGAAGGCCATG	
After	ACCCGGCTCTGGGTAAAGACACCGTTGCTGTTTCTGGTAAATGGTACCTGAAAGCTATG	
	T P A L G K D T V A V S G K W Y L K A M	
	121	180
Before	ACAGCAGACCAGGAGGTGCCTGAGAAGCCTGACTCAGTGACTCCCATGATCCTCAAAGCC	
After	ACCGTGACCAAGGAAGTTCCGGAAAAACCGGACTCTGTTACCCCGATGATCCTGAAAGCT	
	T A D Q E V P E K P D S V T P M I L K A	
	181	240
Before	CAGAAGGGGGCAACCTGGAAGCCAAGATCACCATGCTGACAAATGGTCAGTGCCAGAAC	
After	CAGAAAGGTGGTAACCTGGAAGCTAAATCACCATGCTGACCAACGGTCAGTGCCAGAAC	
	Q K G G N L E A K I T M L T N G Q C Q N	
	241	300
Before	ATCACGGTGGTCCTGCACAAAACCTCTGAGCCTGGCAAATACACGGCATAACGAGGGCCAG	
After	ATCACCGTTGTTCTGCACAAAACCTCTGAACCGGGTAAATACACCGCTTACGAAGGTCAG	
	I T V V L H K T S E P G K Y T A Y E G Q	
	301	360
Before	CGTGTGCTGTTTCATCCAGCCGTCCTCCGGTGAGGGACCACTACATTCTCTACTGCGAGGGC	
After	CGTGTGTTTTTCATCCAGCCGTCCTCCGGTTCGTGACCACTACATCCTGTACTGCGAAGGT	
	R V V F I Q P S P V R D H Y I L Y C E G	
	361	420
Before	GAGCTCCATGGGAGGCAGATCCGAATGGCCAAGCTTCTGGGAAGGGATCCTGAGCAGAGC	
After	GAACTGCACGGTCGTCAGATCCGTATGGCTAACTGCTGGGTCGTGACCCGGAACAGTCT	
	E L H G R Q I R M A K L L G R D P E Q S	
	421	480
Before	CAAGAGGCCTTGGAGGATTTTCGGGAATTCTCAAGAGCCAAAGGATTGAACCAGGAGATT	
After	CAGGAAGCTCTGGAAGACTTCCGTGAATTCTCTCGTGCTAAAGGTCTGAACCAGGAAATC	
	Q E A L E D F R E F S R A K G L N Q E I	
	481	525
Before	TTGGAACCTGCGCAGAGCGAAACCTGCTCTCCAGGAGGACAGTAG	
After	CTGGAACCTGGCTCAGTCTGAAACCTGCTCTCCGGGTGGTCAGTAG	
	L E L A Q S E T C S P G G Q	

(b)

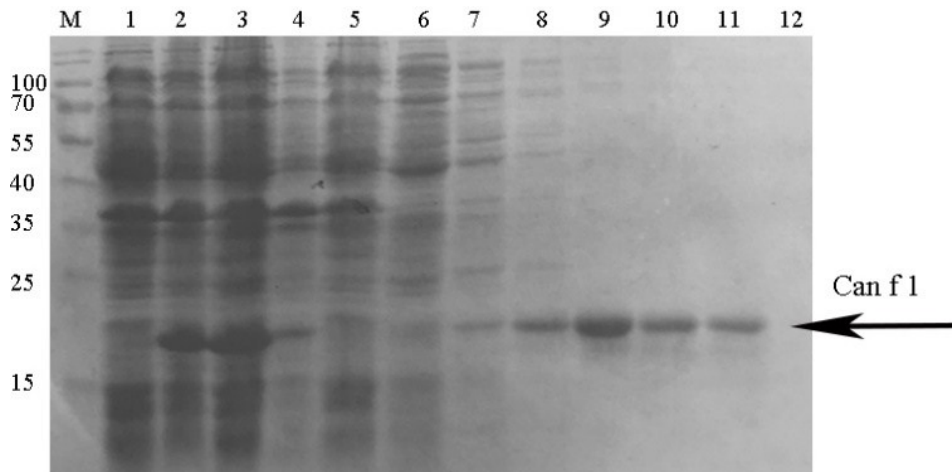


Fig. S2. Expression and purification of Can f 1 in *E. coli*. (a) The nucleotide sequences for codon-optimized Can f 1 gene before and after codon optimization. The underlined part is the codon of signal peptide, which is removed from the sequences when optimized. (b) Lane M, protein molecular weight standard; Lane 1, non-induced recombinant Can f 1 whole cell lysate; Lane 2, IPTG-induced recombinant Can f 1 whole cell lysate; Lane 3, supernatant fraction after ultrasonication; Lane 4, precipitation fraction (inclusion bodies) after ultrasonication. The Can f 1 was denoted with an arrow; Lane 5, unbinding fractions of the supernatant of Can f 1; Lane 6, washing with 10 mM imidazole; Lane 7, washing with 30 mM imidazole; Lane 8, washing with 50 mM imidazole; Lane 9, washing with 100 mM imidazole; Lane 10, washing with 150 mM imidazole; Lane 11, washing with 200 mM imidazole; Lane 12, washing with 250 mM imidazole.

Table S1 Information of 10 patients

No.	Gender	Age	Associated disease	ELISA
1	Female	55	Rhinitis, Eczema	+
2	Male	50	Rhinitis, Eczema	+
3	Female	53	Rhinitis	-
4	Male	15	Rhinitis, Asthma	+
5	Female	35	Bronchial asthma	+
6	Female	21	Rhinitis, Asthma	-
7	Female	29	Rhinitis, Food allergy	+
8	Female	26	Asthma	-
9	Female	27	Bronchial asthma, Rhinitis	+
10	Male	23	Rhinitis, Bronchial Asthma	-

Noted information: +, positive; -, negative

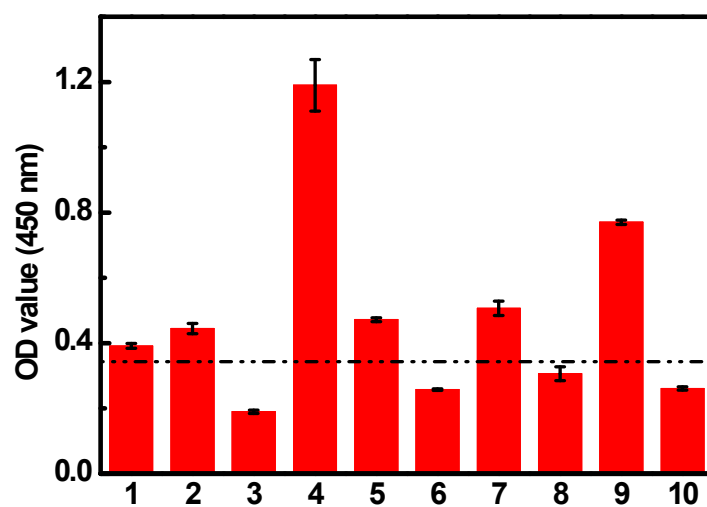


Fig. S3. Detection of Can f 1 sIgEs in 10 patients' sera by commercial ELISA kit. Dotted line indicated the 2.1 times of mean OD value of 5 NHS. All experiments were conducted in quadruplicate.

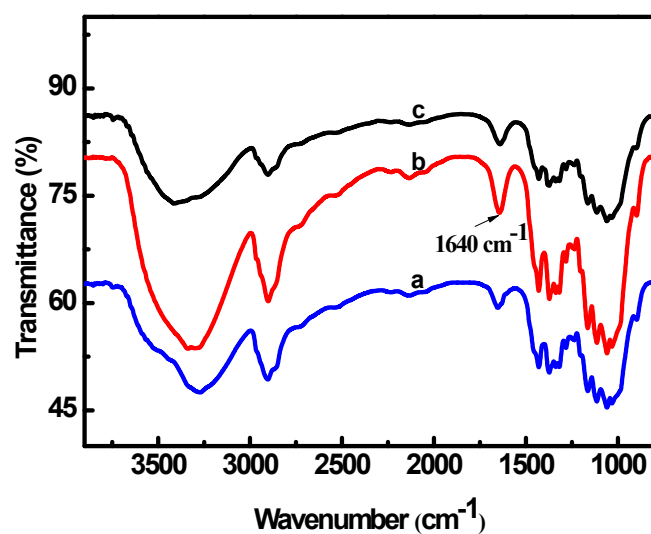


Fig. S4. FT-IR spectra of (a) the original paper; (b) plasma treated paper; (c) the antigen immobilized paper.

H₂O₂

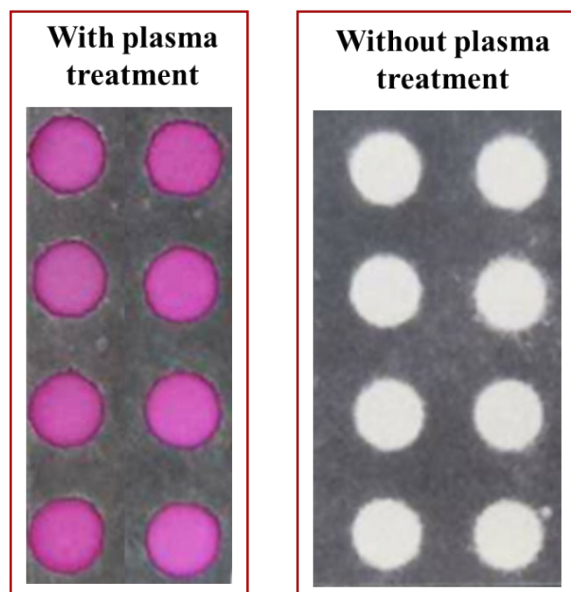


Fig. S5. Verification of aldehyde groups on paper with the Schiff reagent before and after the plasma treatment.

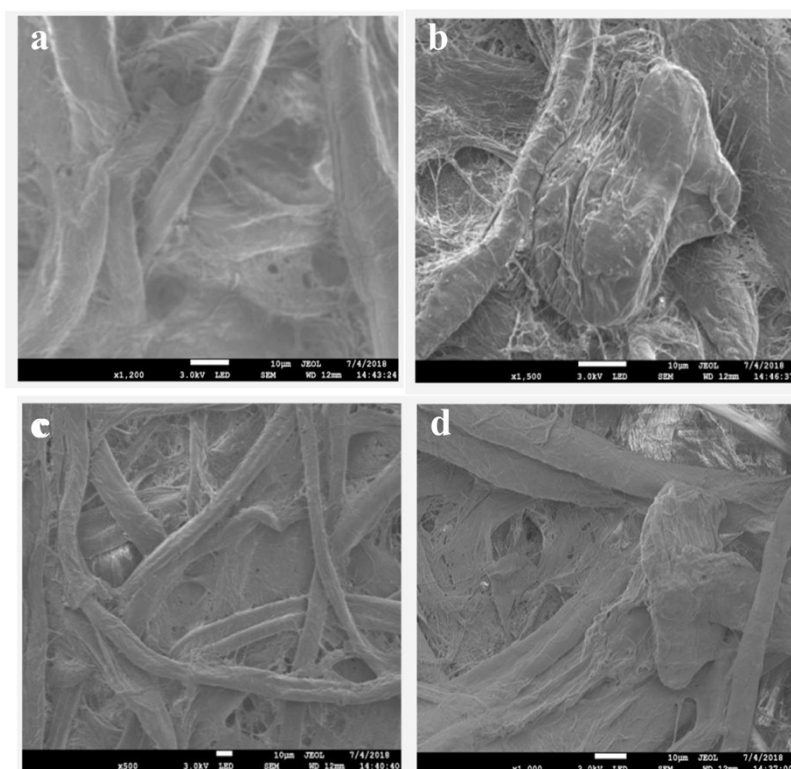


Fig. S6. SEM images showed the characteristics of the paper device before/after oxygen plasma treatment and antibody immobilization. (a) Original paper without treatment; (b) oxygen plasma treated paper; (c) the antigen immobilized paper; and (d) the BSA immobilized paper. Scale bar = 10 μ m.

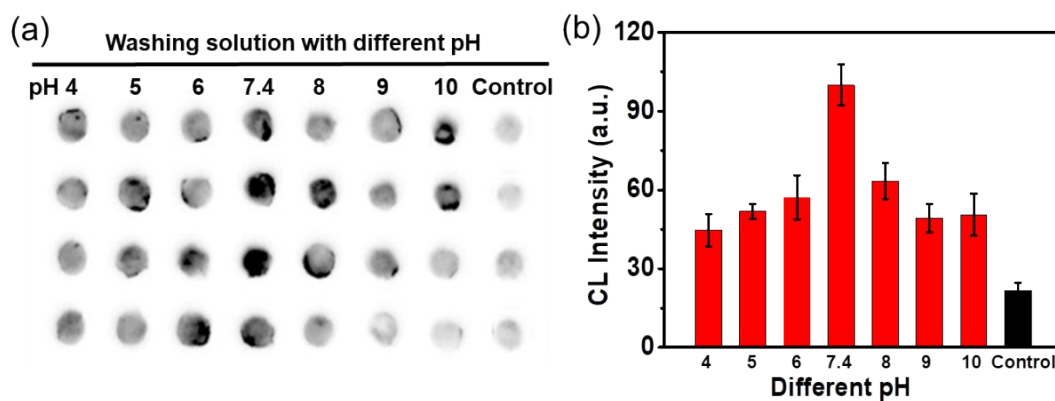


Fig. S7. (a) The CL images of the serum sample washed with different pH buffer solution. (b) The corresponding CL intensity of (a). All experiments were conducted in quadruplicate.

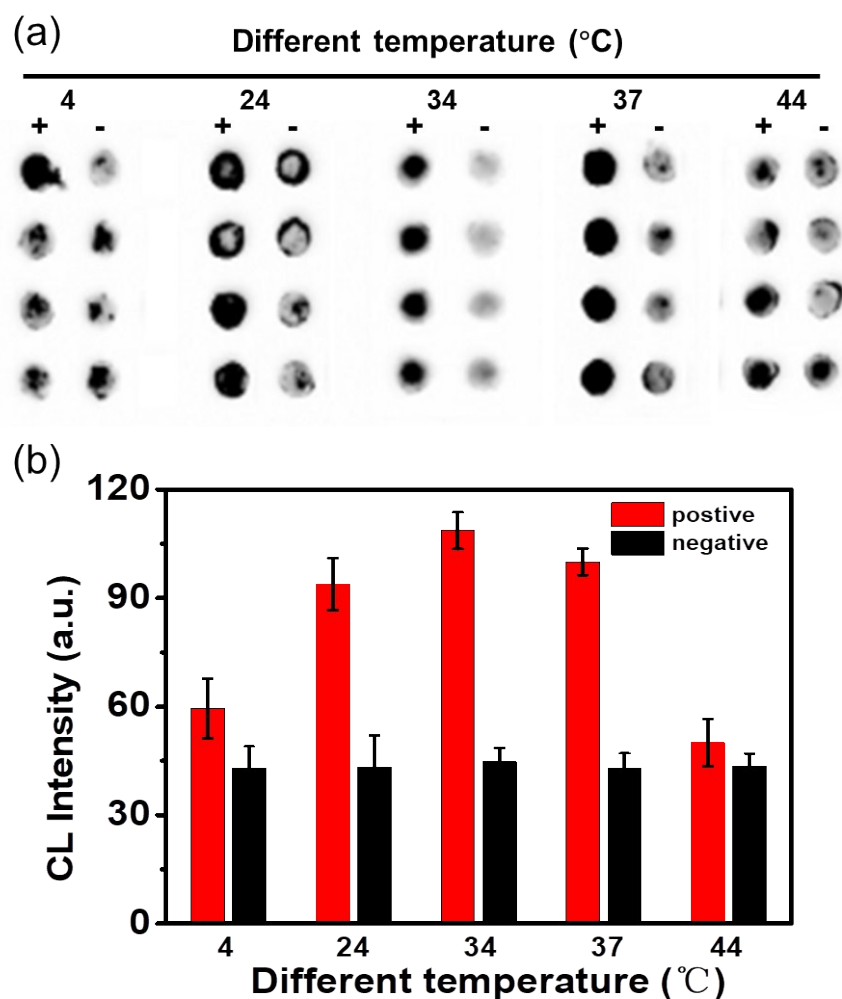


Fig. S8. (a) The CL images of the positive and negative serum sample incubated with different temperature. (b) The corresponding CL intensity of (a). All experiments were conducted in quadruplicate.

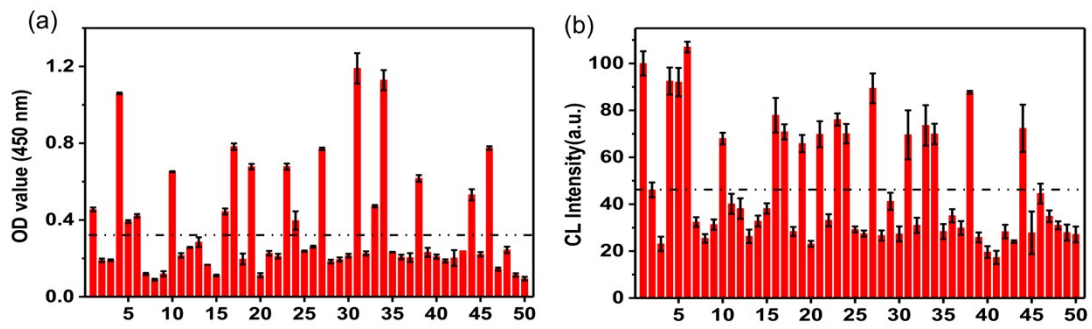


Fig. S9. The OD values at 450 nm of 50 serum samples determined by ELISA kit. Dotted line indicated the 2.1 times of mean OD value of 5 NHS; The CL intensity of 50 serum samples determined by the paper-based device. Dotted line indicated the 1.6 times of mean CL intensity of 5 NHS. All experiments were conducted in quadruplicate.

Table S2. The detailed information of human serum samples and their detection results with commercial ELISA kit and our paper-based device.

No.	Gender	Age	Associated disease	Results	
				ELISA	paper-based device
1	Male	57	Atopic dermatitis	+	+
2	Male	60	Eczema, Cough	-	-
3	Female	53	Rhinitis	-	-
4	Male	56	Rhinitis	+	+
5	Female	55	Rhinitis, Eczema	+	+
6	Male	52	Rhinitis, Cough	+	+
10	Female	19	Rhinitis, Asthma	+	+
11	Male	48	Asthma, Atopic dermatitis	-	-
12	Female	21	Rhinitis, Asthma	-	-
13	Female	35	Rhinitis	-	-
14	Male	14	Rhinitis, Cough	-	-
16	Male	50	Rhinitis, Eczema	+	+
17	Male	19	Rhinitis, Dyspnea	+	+
18	Female	33	Urticaria	-	-
19	Female	17	Rhinitis, Cough	+	+
21	Female	33	Chronic urticaria	-	+
23	Male	43	Conjunctivitis, Cough	+	+
24	Female	27	Bronchial asthma, Rhinitis	+	+
25	Male	27	Hay fever.	-	-
26	Female	26	Asthma	-	-
27	Female	15	Rhinitis, Bronchial asthma	+	+
31	Male	15	Rhinitis, Asthma	+	+
32	Female	32	Rhinitis	-	-
33	Female	35	Bronchial asthma	+	+
34	Female	13	Rhinitis, Asthma	+	+

35	Male	23	Rhinitis, Bronchial Asthma	-	-
36	Female	50	Atopic dermatitis	-	-
38	Male	30	Atopic dermatitis	+	+
44	Female	26	Rhinitis, Bronchial asthma	+	+
45	Male	31	Rhinitis	-	-
46	Female	13	Rhinitis, Asthma	+	-
48	Male	29	Bronchial asthma, Rhinitis	-	-
The rest	Healthy people without a history of allergic symptoms			-	-

Noted information: +, positive; -, negative.

Table S3. The accuracy for each microzone in one sample with the paper-based device. All experiments were conducted in quadruplicate.

Samples	1 (+)	2 (+)	3 (-)	4 (+)	5 (+)	6 (+)	7 (-)	8 (-)	9 (-)	10 (+)
+	70%	67%		72%	100%	87%				64%
-			100%				100%	83%	100%	
Samples	11 (-)	12 (-)	13 (-)	14 (-)	15 (-)	16 (+)	17 (+)	18 (-)	19 (+)	20 (-)
+						67%	75%		88%	
-	83%	71%	89%	88%	63%			89%		100%
Samples	21 (+)	22 (-)	23 (+)	24 (+)	25 (-)	26 (-)	27 (+)	28 (-)	29 (-)	30 (-)
+	75%		83%	61%			75%			
-		100%			91%	91%		71%	78%	67%
Samples	31 (+)	32 (-)	33 (+)	34 (+)	35 (-)	36 (-)	37 (-)	38 (+)	39 (-)	40 (-)
+	67%		73%	56%				81%		
-		80%			75%	70%	60%		100%	67%
Samples	41 (-)	42 (-)	43 (-)	44 (+)	45 (-)	46 (+)	47 (-)	48 (-)	49 (-)	50 (-)
+				71%		64%				
-	67%	100%	100%		67%		71%	70%	92%	67%

Noted information: +, positive; -, negative.