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A light-initiated chemiluminescent assay for rapid quantitation of allergen-specific IgG_4 in clinical samples

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ABSTRACT

Background: An increase in allergen-specific IgG_4 ($sIgG_4$), which serves as a blocking antibody, is associated with acquisition of immune tolerance after immunotherapy. In this study, we developed a rapid, sensitive, and homogeneous immunoassay based on the light-initiated chemiluminescent assay (LICA) technology for quantifying allergen $sIgG_4$ in serum samples.

Methods: Allergen $sIgG_4$ was measured in vitro by incubating the sample with biotinylated allergens and chemiluminescent beads coated with anti-human IgG_4 antibody, followed by the addition of streptavidin-coated sensitizer beads. Multiple tests were performed to optimize the working conditions of the LICA and evaluate its performance.

Results: We established the optimal concentration of biotinylated allergens (250 ng/mL), the optimal dilution range (1:8 for Gal d 1, Gal d 2 $sIgG_4$ and 1:4 for Gal d 3, Gal d 4 $sIgG_4$), and the optimal incubation time (20 min for Gal d 1, Gal d 2 $sIgG_4$ and 40 min for Gal d 3, Gal d 4 $sIgG_4$). The lower limit of quantification (LLOQ) was 0.261 ng/mL. The coefficient variation (CV) of the LICA was < 10%. The assay was unaffected by general interfering substances at physiological concentrations.

It exhibited excellent accuracy to detect allergen-$sIgG_4$ in human serum. Additionally, we demonstrated that the levels of Gal d 1, Gal d 2, and Gal d 3-$sIgG_4$ were significantly higher in the egg allergy group ($p < .05$), but no differences were found between the groups for Gal d 4-$sIgG_4$.

Conclusions: The LICA demonstrated satisfactory performance and can be used for quantifying allergen $sIgG_4$ in clinical practice.

1. Introduction

Food allergy is common, and previous studies have shown an increased prevalence in the past two to three decades [1,2]. According to guidelines, expert panels, and systematic reviews, the recommended serum immunoassays for diagnosing allergy measure food-specific IgE antibodies [3–5]. Measurement of allergen-specific IgG_4 is not considered a diagnostic tool, as it is an unproven test [6–8]. Previous studies have demonstrated that food-$sIgG_4$ is a normal human response to dietary components rather than disease [6,9]. However, increasing evidence has shown that food $sIgG_4$ has blocking activity by competing with IgE for allergen binding, which can lead to a decrease in IgE-facilitated antigen presentation [10–12]. In addition, the development of food tolerance, both natural and oral immunotherapy, is related to an increase in food $sIgG_4$ [13–16]. Children with hen's egg allergy who have a restored clinical tolerance to baked egg are characterized by lower titers of sIgE and up-regulated $sIgG_4$ concentrations [17]. High serum levels of food sIgG4 are associated with allergic children who maintain tolerance to corresponding foods early [18]. In recent years, component-resolved diagnostics (CRDs) have entered into clinical practice and further have improved the specificity and accuracy of the diagnosis of allergy [19]. Rather than relying on crude extract preparations, CRDs measure serum antibodies against specific proteins in food. Studies of CRDs have reported that the measurement of $sIgG_4$ or $sIgE/sIgG_4$ ratio against allergen components contributes to the prediction of tolerance development [20,21]. Therefore, allergen components-$sIgG_4$ are beneficial for monitoring immunotherapy.

Current analytical methods for $sIgG_4$ include enzyme-linked immunosorbent assays (ELISA) and fluorescent immunoassays (FEIA) [21,22]. These assays have intrinsic limitations and require multiple washing steps, a large sample volume, a long immunoreaction time, and are heterogeneous methods that require immobilization of the solid

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phase. Although the quantitative determination kit (ImmunoCAP) for $sIgG_4$ with fluorescent immunoassay developed by Thermo Fisher Scientific is often used, it has not yet been introduced into China. Therefore, a domestically produced immunoassay kit for the robust, sensitive, and rapid detection and quantitation of $sIgG_4$ in serum samples is in urgent demand.

Chemiluminescence (CL) has been widely used in clinical diagnosis and research in recent years, as it provides significant improvement in sensitivity, rapidity, and dynamic range [23,24]. Light-initiated chemiluminescent assay (LICA), which is derived from the luminescent oxygen channeling immunoassay (LOCI) with the desired features, is emerging in China [25]. Similar to the principle of LOCI, LICA is a homogenous bead-based technology in which the proximity of chemibeads and sensibeads (< 200 nm) generates light through CL [26,27].

Hen's egg is one of the most prevalent allergenic food and mainly includes four allergen components: ovomucoid (Gal d 1), ovalbumin (Gal d 2), conalbumin (Gal d 3), and lysozyme (Gal d 4) [28]. In this study, we report the development of a homogeneous immunoassay for the measurement of egg allergenic component-$sIgG_4$ based on LICA technology. For this purpose, we have determined the optimal working conditions, have performed analytical validations, and have evaluated its application.

2. Materials and methods

2.1. Samples

Clinical serum samples ($n = 74$) were obtained from Academy of Traditional Chinese Medicine Affiliated Hospital, China. The samples comprised 42 patients with hen's egg allergy, 16 atopic individuals without a history of egg allergy, and 16 non-atopic healthy subjects. The diagnosis of IgE-mediated hen's egg allergy was based on a convincing clinical history related to egg consumption, severe and/or acute reactions, and increased specific IgE levels (egg-sIgE > 0.35 kU_A/L) measured using the CAP System FEIA. The clinical characteristics of the studied subjects are given in Table 1. Written informed consent was obtained before patient enrollment. The study was approved by the Ethics Committee of Tianjin Medical University (TMUHMEC2017008) and conducted in accordance with the principles of the Declaration of Helsinki.

2.2. Material and chemicals

Commercially available egg allergenic components and interfering substances were purchased from Sigma-Aldrich (St. Louis, MO, USA). Unconjugated chemibeads and streptavidin-coated sensitizer beads were obtained from Beyond Biotech (Shanghai, China). Primary human purified IgG4 with a purity of > 95% by SDS-PAGE analysis and secondary mouse anti-human IgG4 antibody were purchased from Abcam (Cambridge, UK). EZ-LinkTM Sulfo-NHS-LC-LC-Biotin was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Signals were measured using a CL analyzer from Beyond Biotech (Shanghai, China).

Table 1
Clinical characteristics of the studied subjects.

Patient characteristics	HEA ($n = 42$)	Non-HEA ($n = 32$)
Age (years)	22 (4–37)	19 (2–41)
Gender (male/female)	19/23	18/14
Total serum IgE mean and range (kU/L)	80 (6–765)	23 (1–206)
sIgE to egg mean and range (kU_A/L)	7.32 (0.36–30.46)	< 0.35
Symptoms (most frequent only)		
With egg intake cutaneous (%)	30 (71.4)	0(0)
Cutaneous + gastrointestinal (%)	19 (45.2)	0(0)
Other allergies, number (%)	13 (27.1)	16 (50)

2.3. Biotinylated allergen

Egg white allergen proteins were biotinylated according to standard procedures for biotinylating proteins in solution. Briefly, allergens were dissolved in 0.01 M phosphate-buffered saline (PBS, pH 7.4). The appropriate volume of biotin reagent solution was added to the protein solution at a molar ratio of 20:1 and incubated on ice for 2 h. Calculations were performed based on the product introduction. To remove unreacted biotin, the solution was desalted or dialyzed against PBS buffer at 4 °C. Finally, the conjugate was stored at 4 °C until further use.

2.4. Coupling of anti-IgG_4 to beads

Two milligrams of chemibeads were washed twice with ultrapure water before use and then resuspended in 0.05 M carbonate buffer (pH 9.6) by vortexing for 1 min and sonicating for 30 s. The solution was added to 0.1 mg of anti-human IgG4 antibody and incubated overnight at 37 °C in a rotary shaker. The next day, the reaction mixture was added to 10 μL of 8 mg/mL $NaBH_4$ and then incubated for 2 h at room temperature (RT). Afterwards, 40 μL of 75 mg/mL glycine was added to block the free sites on beads and the solution stirred for 1 h at RT. Finally, the conjugate was centrifuged and washed, and then the remaining pellet resuspended and preserved in 200 μL of storage buffer (20 mM HEPES +0.2% BSA + 0.1% Proclin-300).

2.5. Preparation of IgG_4 standards

The human purified IgG_4 was biotinylated in accordance with standard procedures as described above. The assay buffer was 0.01 M PBS (pH 7.4). For calibration purposes, the calibrator was serially diluted in assay buffer to generate the standard concentrations of 0, 0.05, 0.5, 5, 50, 500, 5000, and 50,000 ng/mL. The prepared standard solutions were stored at 4 °C.

2.6. LICA procedure

The principle of LICA for the quantitation of $sIgG_4$ is illustrated in Fig. 1. This proposed immunoassay followed a two-step reaction. The experiments were performed with calibrators and samples in duplicate. For the general assay procedures, test serum samples were added to the wells of a 96-well microtiter plate. A mix of 25 μL of anti-human IgG_4 antibody-coupled chemibeads and 25 μL of biotinylated allergens were then added. For the standard curve, anti-human IgG_4 antibody-coupled chemibeads in a volume of 25 μL were added and incubated with 25 μL of 10-fold serial dilutions of the calibrator. The plates were incubated at 37 °C for 20 min with mild agitation on a shaking platform. Finally, the reaction mixture in all wells was added to 150 μL of streptavidin-coated sensibeads and incubated at 37 °C for another 10 min under dark conditions. The CL intensity was then measured on the LICA reader.

2.7. Statistical analysis

Differences between groups were analyzed using the independent test for parametric data and the Mann-Whitney U test for non-parametric data. $P < .05$ was considered significant. All statistical analyses were performed using GraphPad Prism Version 5.0 software.

3. Results

3.1. $sIgG_4$-LICA optimization

Immunoassay sensitivity was affected by multiple factors. Multiple tests were performed to optimize the working conditions of the LICA, including the concentrations of biotinylated antigens, the dilution ratio of serum, and the incubation time of the first reaction step (Fig. 2). Gal

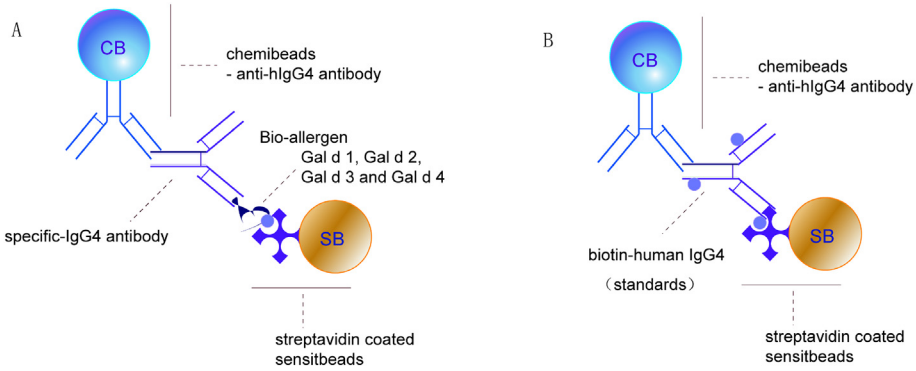

Fig. 1. Schematic diagram of LICA. (A) For the detection of allergen-sIgG$_4$. (B) For the calibration curve.

d 1, the most allergenic hen's egg component, was selected to optimize reaction conditions and for further analytical validation. Three serum pools with low, medium, and high levels of Gal d 1 sIgG$_4$ were collected and quantified by the present homogeneous CL assay. The CL intensity was expressed as counts per second (cps).

3.1.1. Optimal concentration of biotinylated antigens for sIgG$_4$-LICA

The concentration of biotinylated antigen was an important parameter for immunoassay sensitivity. Insufficient antigen was expected to have an adverse effect on CL intensity and sensitivity. However, excessive antigen may lead to the hook effect. Biotin-Gal d 1 was gradually diluted from 2000 ng/mL to 67.5 ng/mL in assay buffer. When the concentration of biotin-Gal d 1 was 250 ng/mL, the CL signal was maximized (Fig. 2A). Thus, biotinylated allergen at a concentration of 250 ng/mL was enough to bind with sIgG$_4$ in serum. Therefore, in subsequent experiments, 250 ng/mL of biotinylated allergens was used.

3.1.2. Optimal serum dilution for sIgG$_4$-LICA

To determine the optimal sample dilutions, three serum pools with low, medium, and high levels of Gal d 1 sIgG$_4$ were tested in serial dilutions from 1:2 to 1:16 (Fig. 2B). For serum pools with medium and high levels of sIgG4, the CL intensity increased with increasing serum dilutions from 1:2 to 1:8. For the serum pool with low levels of sIgG$_4$, the reaction reached equilibrium status when the serum dilution was 1:4. As levels of Gal d 1 and Gal d 2 sIgG$_4$ were higher in subsequent sample tests and levels of Gal d 3 and Gal d 4 sIgG$_4$ were lower, to obtain the best CL signal, we chose 1:8 serum dilutions for the Gal d 1, Gal d 2 sIgG$_4$ assay and 1:4 for the Gal d 3, Gal d 4 sIgG$_4$ assay.

3.1.3. Optimal incubation time for sIgG$_4$-LICA

The interaction time for immunoreagents in the immunoassay has been reported to affect the sensitivity of the assay [29]. Therefore, we ranged the time of the reaction step between antigen and antibody from 5 to 40 min (Fig. 2C). For serum pools with medium and high levels of sIgG$_4$, the CL intensity peaked near 20 min. For the serum pool with low levels of sIgG$_4$, the CL intensity increased with longer incubation time. Similar to the principle of optimizing serum dilutions, the reaction time was 20 min for the Gal d 1, Gal d 2 sIgG$_4$ assay and 40 min for the Gal d 3, Gal d 4 sIgG$_4$ assay.

3.2. sIgG$_4$-LICA validation

According to the requirements and acceptance criteria for bioanalytical method validation [30,31], we performed a number of

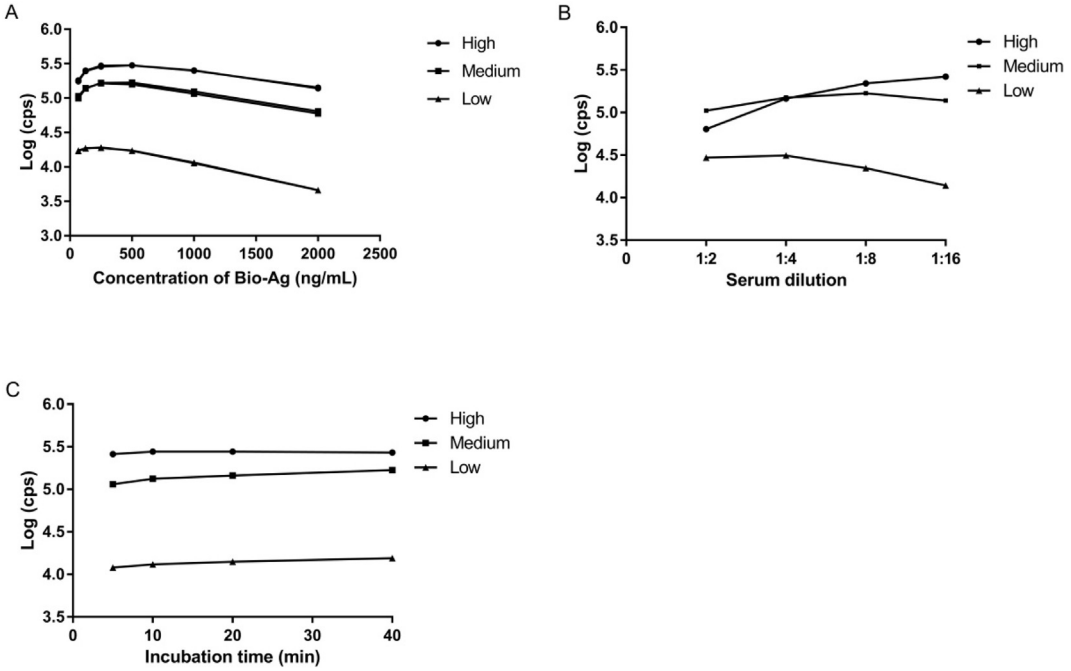

Fig. 2. Optimization of the working conditions for the LICA. (A) Effect of biotin-Ag concentration on LICA signal. (B) Effect of serum dilution on LICA signal. (C) Effect of incubation time on LICA signal. The error bars were invisible because they were shorter than the height of the symbol.

experiments to verify the performance of this immunoassay for the detection of sIgG4 in terms of sensitivity, precision, accuracy, and anti-interference.

3.2.1. Calibration curve

A standard dose-response curve ($Y = 0.755\times + 3.498$, $R^2 = 0.990$) was constructed (Fig. S1) using linear regression on a logarithmic plot with six calibration concentrations, including zero calibrator (IgG_4 concentration of 0 ng/mL standard solution). For the calibration curve depicted in Fig. S1A, the intensity range was 330 to 319,187 cps from the zero calibrator to 500 ng/mL. When the range of IgG_4 exceeded 5000 ng/mL, signal intensity saturation or the hook effect was clearly observed (Fig. S1B). Furthermore, the zero calibrator was tested 10 times. The limit of detection (LOD) was 0.051 ng/mL based on the dose-response curve and the mean plus 2 standard deviations of the zero calibrator.

3.2.2. Sensitivity

The lower limit of quantification (LLOQ) was defined as the lowest sIgG4 concentration that could be quantitatively determined with acceptable accuracy and precision and an interassay coefficient of variation (CV) < 20%. The CV was calculated using the following formula: CV (%) = (SD /mean) × 100. To assess this parameter, the high Gal d 1 $sIgG_4$ sample was serially diluted in assay buffer and each dilution tested six times on separate days. The LLOQ was 0.261 ng/mL (Fig. S2).

3.2.3. Precision

As an important index for quantitative experiments, assay precision was evaluated by calculating the inter-batch and intra-batch CVs. To determine the interassay variation, 10 duplicates of each sample were analyzed in parallel in the same run. In the intraassay study, the variation was evaluated by analyzing the same homogenous samples under the prescribed conditions for 10 consecutive days, one duplicate per day. The precision results are shown in Table 2. The intraassay CV% varied from 2.7% to 5.1%, and the interassay CV% was between 4.23% and 9.62%, suggesting that the results of this assay had satisfactory reproducibility.

3.2.4. Accuracy

The accuracy was evaluated by linearity under dilution. The dilution linearity of this assay was determined using two samples serially diluted with assay buffer (1:8, 1:16, 1:32, 1:64, 1:128, 1:256). Expected values were calculated from the concentrations of $sIgG_4$ in the initial diluted samples. Subsequently, curves representing measured values versus expected values were obtained using linear regression. The r values were 0.999 and 0.998 for samples 1 and 2, respectively (Fig. S3), which indicates that the concentration of $sIgG_4$ was accurately measured in the samples.

3.2.5. Interference

Interference testing was performed by the addition of sample matrix components to the serum samples at the stated final concentrations. The recovery (> 80%) was acceptable. As shown in Table 3, hemolysate up to at least 20 μmol/L, bilirubin up to at least 100 μmol/L, triglyceride up to at least 14,000 μmol/L, biotin at a concentration of < 0.01 μmol/L, and ascorbic acid < 1000 μmol/L did not significantly affect the test results.

Table 2
Evaluation of within-and between-runs precision.

Serum pool (ng/ml)	Interassay (n = 10)			Intraassay (n = 10)		
	Mean	SD	CV(%)	Mean	SD	CV(%)
Low	8.04	0.77	9.62	9.74	0.26	2.64
Medium	171.97	12.61	7.33	191.15	7.38	3.86
High	404.22	17.11	4.23	420.16	9.59	2.28

Table 3
Interference test of LICA for $sIgG_4$.

Interfering substance	Glad 1-sIgG4 (ng/ml)	Recovery (%)
Hemoglobin (μmol/L)		
0	9.80	100
10	9.11	93
20	8.61	87.9
Bilirubin (μmol/L)		
0	10.20	100
50	9.62	94.3
100	8.77	86
Triglyceride (μmol/L)		
0	188.09	100
7000	199.34	106
14,000	168.43	89.5
Biotin (μmol/L)		
0	160.46	100
0.01	140.59	87.6
0.05	112.07	69.8
Ascorbic acid (μmol/L)		
0	431.66	100
1000	391.05	90.6
5000	303.25	70.3

3.3. Analysis of study samples

Next, we measured the levels of sIgG4 in the study samples using the homogeneous chemiluminescent assay system under optimum conditions. The levels of Gal d 1, Gal d 2, and Gal d 3-$sIgG_4$ were significantly higher in the egg allergy group ($p < .05$), but no differences were found between the groups for Gal d 4-$sIgG_4$ (Fig. 3). In addition, in each group, levels of Gal d 1, Gal d 2-$sIgG_4$ tended to be much higher than the levels of Gal d 3, Gal d 4-$sIgG_4$.

4. Discussion

In this study, we established a homogeneous serological assay for the rapid quantitation of allergen-$sIgG_4$. As with LOCI or AlphaScreen technology [32,33], this assay involves two types of polystyrene beads, chemibeads and sensibeads. Sensibeads contain a photosensitizer, phthalocyanine, which converts adjacent ambient oxygen to an excited form of singlet oxygen upon laser excitation at 680 nm. The singlet oxygen molecules diffuse across to react with thioxene derivatives within the chemibeads, thereby generating CL at 520–620 nm. The singlet oxygen has a short life span of 4 μs and can effectively migrate a distance of approximately 200 nm during this period. If these two types of beads approach each other within the range of biological interaction of antigen-antibody and biotin-streptavidin, light will be emitted.

LICA is a mix-and-measure assay. Compared to other analytical methods, this assay does not require separation or multiple washing steps prior to the addition of reagents, thus realizing rapid quantitation of allergen-$sIgG_4$ within 1 h.

In addition to rapid turnaround cycles, the $sIgG_4$-LICA is very sensitive, with a LOD of 0.051 ng/mL and LLOQ of 0.261 ng/mL based on results from calibrators and samples, respectively, allowing quantitation of low concentrations of allergen-$sIgG_4$. In addition, as seen from the calibration curve, when the amount of anti-IgG_4 antibodies is constant, the reaction reaches equilibrium status at a calibrator concentration of 5000 ng/mL. Consequently, the upper limit of quantification (ULOQ) is 5000 ng/mL. The broad analytical range of the assay makes it suitable for detecting samples with a variety of $sIgG_4$ concentrations.

As the LICA requires no separation or multiple washing steps, it may be more sensitive to interfering components in serum samples. The general interfering substances in serum include hemoglobin

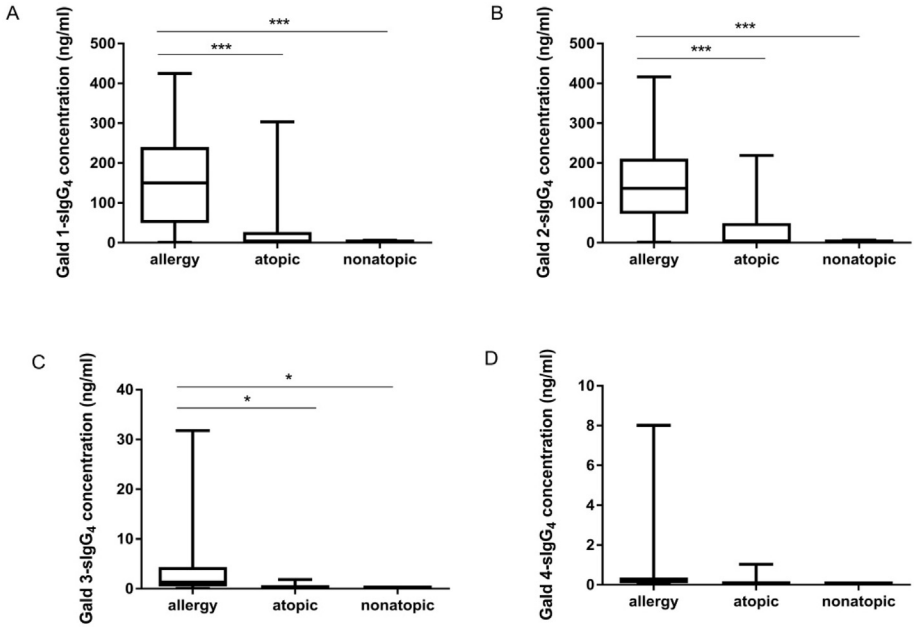

Fig. 3. Detection of allergen $sIgG_4$ using LICA. The whiskers represent data are presented from minimum to maximum. * $p < .05$, *** $p < .001$.

(hemolysis), bilirubin, triglyceride, ascorbic acid, and biotin. The main potential singlet oxygen quenchers are ascorbic acid and heme iron. High levels of ascorbic acid and heme iron have both been suggested to have an inhibitory impact on the LICA [34,35]. Our results demonstrate that the assay was unaffected by ascorbic acid and heme iron at physiological levels in serum. Moreover, because the LICA is a biotin–streptavidin system, the assay results may be adversely impacted by biotin. Previous studies have reported biotin interference in immunoassays [36,37]. The present results indicate that biotin at physiological concentrations in serum do not disrupt the assay. However, an increase in biotin would result in a significant reduction of CL intensity.

The applicability of the assay for $sIgG_4$ was investigated using patient and control sera. Our results revealed significantly higher levels of $sIgG_4$ antibodies against Gal d 1 and Gal d 2 among patients with an egg allergy than against Gal d 3 and Gal d 4. Studies have reported that Gal d 1 is the most allergenic component in eggs, and Gal d 2 is the most abundant protein in egg white [38]. This information combined with our present findings suggests that Gal d 1 and Gal d 2 have stronger antigenicity and allergenicity than Gal d 3 and Gal d 4. In additionally, Tay et al. found that serum levels of Gal d 1 sIgG and $sIgG_4$ are almost identical in allergic, tolerant, healthy subjects [39]. However, the group with egg allergy in the current study had significantly higher mean concentrations of $sIgG_4$ antibodies against Gal d 1, Gal d 2, and Gal d 3 than the non-egg allergic groups. People from different ethnic backgrounds or in different regions may have different tendencies for developing reaction patterns [40].

To the best of our knowledge, this study is the first to describe a homogeneous CL.

immunoassay for quantitation of allergen $sIgG_4$. The LICA for allergen $sIgG_4$ exhibited satisfactory performance with rapid turnaround cycles, high sensitivity, broad analytical range, and excellent reproducibility. We think that this new method will facilitate the immunodetection of allergen $sIgG_4$ and be of high value in applications for monitoring immunotherapy.

Disclosure statement

All authors have declared they have no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2018.11.036.

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