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Triple quantitative detection of three inflammatory biomarkers with a biotin-streptavidin-phycoerythrin based lateral flow immunoassay

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ABSTRACT

Quantified inflammatory biomarkers are effective clinical strategy for correct and reasonable drug treatment. In the study, a triple lateral flow immunoassay (triple LFIA) had firstly been developed for specific and simultaneous detection of three pivotal inflammatory biomarkers (PCT, CRP and SAA) *via* biotin-streptavidin-phycoerythrin signal amplification system in one strip. The developed triple LFIA adopted phycoerythrin (PE) as chromophore to eliminate auto-fluorescence interference from plasma biomolecules and anti-PE mAb as single control line to reduce the nonspecific adsorption, which featured particular advantages in high sensitivity and specificity in a large range of analyte concentrations with the LODs of 0.106 ng/mL for PCT, 0.345 μg/mL for CRP and 3.112 μg/mL SAA, respectively. And the linear quantitative detection ranges were from 0.106 to 100 ng/mL, from 0.345 to 200 μg/mL, and from 3.112 to 200 μg/mL, respectively. Compared to commercial chemiluminescence immunoassay method, the correlations for tested PCT, CRP and SAA in 108 clinical samples were 0.989, 0.987 and 0.988, respectively. In summary, we had proposed a rapid and accurate plasma detection to measure inflammation factors, which facilitated the clinical value to achieve precise treatment.

1. Introduction

Inflammation is a non-invasion pathological process caused by virus or bacterial invasion. The patient with different etiology requires different therapeutic schedule. Inflammation biomarkers, such as procalcitonin (PCT), C-reactive protein (CRP), and serum amyloid A (SAA), are effective clinical strategy for correct and reasonable drug treatment [1–3].

PCT is induced by bacterial endotoxin [4], and the concentration is lower than 0.25 ng/mL in the healthy blood and can rise up to 100 ng/mL for systematic bacterial infection [5,6]. PCT concentration can also increase in non-infection conditions (eg. surgery and surgical trauma) but not changed significantly with viral infection, so it may not be enough based on PCT alone to diagnose invasive bacterial or viral infection and the severity assessment. CRP is the first acute reactive protein to be discovered [7], and the concentration is elevated in response to bacterial invasion or tissue damage from less than 5 μg/mL in healthy plasma to 40–200 μg/mL under bacterial infections. SAA is mainly produced in the liver as an acute phase protein, and is a sensitive indicator of early inflammation in infectious diseases, which rapid increases in both bacterial and viral infections [8,9]. For the patient with viral infection, SAA level increases more markedly than the plasma CRP level [10]. Serum PCT also rises rapidly than CRP and reaches peak within very short time. Therefore, it is unlikely that a single biomarker serves as an effective diagnosis tool to differentiate bacterial or non-bacterial infection. Simultaneous and rapid detection of PCT, CRP and SAA can be helpful in (i) differentiating bacterial infection from non-infective causes of inflammation; (ii) differentiating acute from chronic bacterial infection as well as local from systematic bacterial infection; (iii) and furthermore, determining the appropriate dosage and duration of antibiotic therapy.

Enzyme-linked immunosorbent assay (ELISA) [11], chemiluminescence immunoassay(CLIA) [12,13], electrochemical luminescence immunoassay (ECLIA) [14], and lateral flow immunoassay (LFIA) are frequently used for determination of inflammatory biomarkers. LFIA is simple operation based on the principles of chromatography to detect

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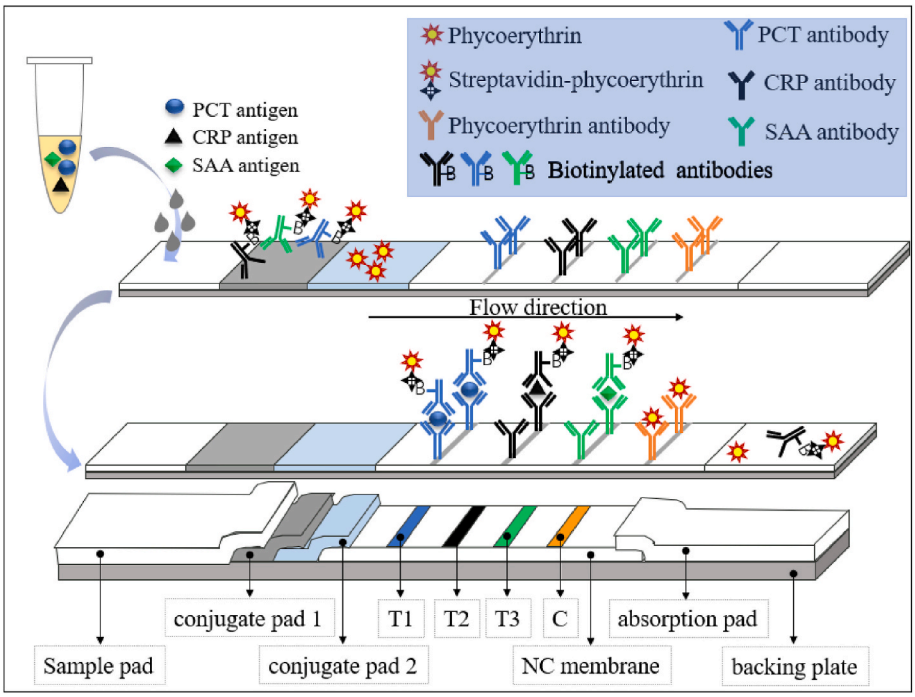


Scheme 1. The principle of LFIA strip to simultaneously measure three inflammatory biomarkers with B-SA-PE signal amplification system.

biomarkers, bacteria, virus and toxin [15–19]. However, multiplex assay can simultaneously analyze or measure several analytes by a single test at the same conditions, which is important for clinical diagnosis to be served as the parameters predicting different stages of disease or unidentified illness. So many efforts have been performed for multiplex detection, like Raman scattering [20], quantum-dot-tagged microbeads [21,22], immunochip [23], colorimetric array test strip [24], multiplex ELSIA [25], and LFIA [26,27]. Due to the advantages of low cost, rapidity, requirement of small amount of sample and other benefits, LFIA is the best choice for multiple analytes to be simultaneously detected. The simultaneous multiple detection of inflammatory biomarkers within a single LFIA test strip could significantly shorten total analysis time, save sample volume, and reduce test cost compared with many individual test strips. However, multiple detection was much more difficult than single detection, not only for the improved demand of instruments but also for the cross interference caused by non-specific interaction and spectral interference. In recent years, some dual detections of inflammatory biomarkers have made great progress, such as upconversion nanoparticle [28], surface enhanced Raman scattering (SERS) nanomaterials [29], and quantum dots (QDs) [30–32]. However, the syntheses of these nanomaterials are rather complex for the multiplex detection of targets in clinical samples. In addition, their poor biocompatibility and the cross interference between different biomarkers had greatly limited their wide application in multi-LFIA system. Phycoerythrin (PE) is a phycobiliprotein with intense fluorescence as bioluminescent probe in immunofluorescence assay [33–35]. Compared with radiative probes and chemiluminescence probes (eg. QD, magnetic QD and colloidal Au), PE has high safety, good biocompatibility and water solubility. The spectral characteristics of PE are almost unchanged in the pH range of 4–11, which is conducive to improve the sensitivity. Thus, PE will have a wide application prospect in clinical diagnosis.

Herein, we want to propose a novel triple LFIA strip with biotin-streptavidin-phycoerythrin (B-SA-PE) signal amplification system for the quantitative and simultaneous detection of three inflammatory biomarkers (PCT, CRP and SAA) in serum samples. In the system, single-color phycoerythrin-based LFIA strip will be developed with unique control line to eliminate experimental error of control line associated with many measurements. The proposed triple LFIA system can be also used for rapid and simultaneous analysis of other types of analytes with the different specific antibodies in the future.

2. Materials and methods

2.1. Chemicals and materials

CRP mAb1 and mAb2 was purchased from HyTest Ltd (Turku, Finland). SAA mAb1 and mAb2 was purchased from Medix Biochemica (Kauniainen, Finland). Streptavidin-Phycoerythrin (SA-PE), PE, PCT mAb1 and mAb2 were provided by Guangzhou Tebsun Bio-Tech Development Co., Ltd. (Guangzhou, China). NHS-LC-Biotin, HT, DMEM and Pen-Strep solution were purchased from Thermo Fisher Scientific Inc (Waltham, USA). Photomultiplier tube SFH 2430 was purchased from OSRAM Opto Semiconductors (Munich, Germany)

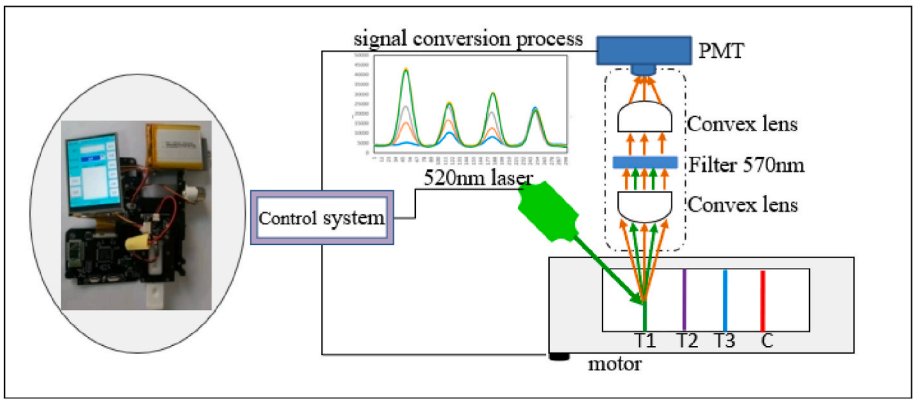
Complete Freund's sadjuvant, incomplete Freund's sadjuvant, polyethylene glycol 1450, DMSO and HAT medium were purchased from Sigma-Aldrich Co. (Louis, USA). Balb/c mice were purchased from the Animal Center of the Chinese Southern Medical University (Guangzhou, China). All other reagents used in this work were of analytical grade. Human plasma samples were obtained from General Hospital of Southern Theatre Command of PLA (Guangzhou, China).

2.2. Self-assembly of antibody conjugates with NHS-LC-Biotin and SA-PE

The mAb1 of PCT, CRP and SAA were dialyzed with PBS (pH 7.4) at 4 °C for 3 times and diluted to 2 mg/mL using PBS (pH7.4) before conjugated. The labeling reaction between mAb1 and NHS-LC-Biotin (B) was performed by following the EZ-Link® NHS-biotin reagent instruction. The dialyzed mAb1 of PCT, CRP and SAA were mixed with NHS-LC-Biotin at 200 μM, 100 μM, 100 μM, and incubated at 30 °C for 30 min to form mAb1-B. NH_4Cl was added with the final concentration of 1 mM to terminate the labeling reaction at 30 °C for 10 min. Then, the mixture was dialyzed with PBS (pH7.4) at 4 °C for 3 times and stored at −20 °C for further use.

mAb1-B-SA-PE.

The conjugates of mAb1-B-SA-PE were obtained by mixing the solution of mAb1-B and SA-PE for 10 min at 37 °C, respectively, where the



Scheme 2. The schematic structure of the fluorescent reader.

final concentration of mAb1-B was adjusted to be 1 mM, and treated with the final NH_4Cl concentration of 1 mM.

2.3. Fabrication of anti-PE monoclonal antibody (Anti-PE mAb)

PE was emulsified in an equal volume of complete Freund's adjuvant. Then, 50 μg of PE was used to immune six-to eight-week-old Balb/c mice. Triple booster immunizations were administered with the same amount of PE in Freund's incomplete adjuvant every 2 weeks. Three days before cell fusion, a final booster was administered *via* intravenously injection at equivalent dose in PBS.

Hybridoma fusion was performed according to method described by Kohler and Milstein [36]. The suspension of spleen cells extracted from immunized mice was mixed with a suspension of Sp2/0 myeloma cells at a cell ratio of 10:1–4:1. The cells were fused by 50% polyethylene glycol 1450 in serum-free DMEM, and hybridomas was cultured in HAT selection medium. After 10 days, positive clones were screened by ELISA using PE as the coating antigen and SA-PE as control. And then, the single positive clone was subcloned for three times to screen out the positive monoclonal antibody with the strongest affinity to PE but non-affinity to SA-PE. The isotype of the monoclonal antibody was performed as instruction described by the commercial kit (Biodragon, Beijing, China). The antibody was purified by the Protein G (GE, St. USA) and determined by sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously with either 15% acrylamide gel or gradient (from 7.5% to 20%) gel [37].

The affinity of anti-PE mAb was assessed with non-competitive ELISA method. Four grades of coating PE concentration were used to establish the standard curve with a series of diluted anti-PE mAb concentrations in the binding reaction. The affinity constant was calculated by equation (1) [38]:

$$K = (n-1)/2(n*mAb'-mAb) \quad (1)$$

K was the antigen-antibody affinity constant in mol^{-1}, n was the ratio of Ag at two different concentrations in the coating solutions, mAb and mAb' were monoclonal antibody concentrations at OD_{50} for plates coated with two amounts of antigen.

2.4. Preparation of triple LFIA strip

Triple LFIA strip consisted of five key parts: the sample pad, conjugate pads 1 and 2, NC membrane, absorption pad and backing plate as shown in Scheme 1. The sample pad and the conjugate pads were treated by soaking in buffer (1 × PBS, 0.5% w/v trehalose, 0.25% w/v PVA, 0.5% w/v BSA, 0.05% v/v Tween-20) and in buffer (1 × PBS,1% w/v trehalose,0.5% w/v PVA,1% w/v BSA,0.1% v/v Tween-20) for 10 min, respectively, and then dried at 40 °C for 10 h. The mAb1-B-SA-PE of PCT, CRP and SAA complexes were evenly sprayed onto conjugate pad 1, while PE was evenly sprayed onto conjugate pad 2 by IsoFlow Dispenser (Imagene Technology, Lebanon, NH, USA) at a jetting rate of 3.9 μL/cm, then dried at 40 °C for 5 h. The mAb2 of PCT, CRP, SAA (1.5 mg/mL) and anti-PE mAb (1.0 mg/mL) were separately spotted onto the NC membrane at a jetting rate of 1.0 μL/cm to generate test line 1 (T1), test line 2 (T2), test line 3 (T3) and control line (C) with leaving about 3 mm between each adjacent two lines, and then dried at 50 °C for 3 h. Subsequently, the sample pad, conjugate pads 1 and 2, NC membrane, and absorption pad were assembled onto a backing plate, and then cut into individual 4 mm wide strip by ZQ4500 cutter (Shanghai Kinbio Tech Co., Ltd, shanghai, China) for subsequent use. The strips were stored in sealed aluminum foil bags with desiccants at room temperature.

2.5. Standard sample assays and the schematic structure of the fluorescent reader

The standard curves of PCT, CRP and SAA were calculated based on three kinds of serial concentrations of standard antigens (0, 0.25, 2, 25, 50, 100 ng/mL for PCT, and 0, 10, 30, 75, 150, 200 μg/mL for CRP and SAA). The cross-interferences of PCT, CRP and SAA were determined by comparing with Test/Control (T/C) ratios of the same serial concentrations of standard antigen mixture.

The determination protocol of standard samples was as follow: 10 μL of each standard sample above was mixed with 490 μL of running buffer (0.05 M Tris-HCl, pH 8.0, 0.9% w/v NaCl, 0.5% w/v BSA), and 100 μL of the corresponding solution was added onto the sample pad of triple LFIA strip at room temperature. The fluorescence intensities (FIs) of the test lines T1, T2, T3 and the control line C were readout by the fluorescent reader designed in our Lab. Each sample was tested for three times and the mean values of T1/C, T2/C and T3/C ratios were plotted against the concentrations of PCT, CRP and SAA, respectively. The corresponding PCT, CRP and SAA concentrations were output after a matching analysis with the standard curve. The schematic structure of the fluorescent reader was depicted in Scheme 2. A HC352010D fibre laser at 520 nm (Maizhi Laser, Zhuhai, China) as the excitation light source could irradiate SA-PE on the test lines and PE on the control line to produce the fluorescence emission at 570 nm which were received by SFH 2430 photomultiplier tube (PMT, OSRAM Opto Semiconductors, Germany) after going through narrow band filter. And the FI distributions along the test strips were recorded *via* an electric rotating motor scan along the strip from T1 line to C line smoothly during the measurement process.

2.6. Method comparison and performance of triple LFIA

To estimate the accuracy and reproducibility of the developed triple LFIA method, intra-assay and inter-assay were applied to measure the precision. Three different samples with three concentrations (0.5, 5 and

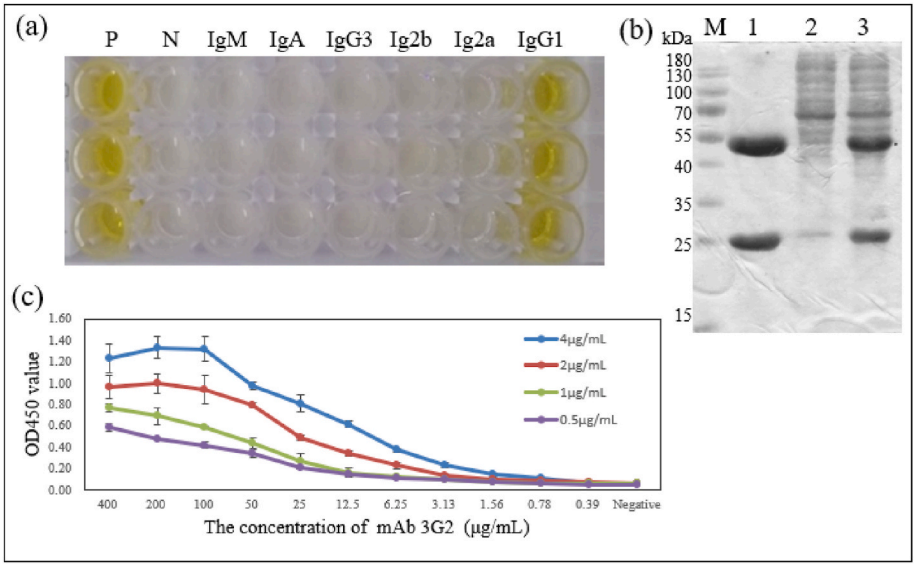


Fig. 1. Performance of anti-PE mAb. a) The mAb 3G2 was of subclass IgG1; b) SDS-PAGE of 3G2. The molecular weight of the heavy chain was about 55 kDa and the light chain was about 25 kDa; c) Response curve of mAb 3G2 at the gradient of Ag (PE).

50 ng/mL for PCT; 5, 50 and 150 μg/mL for CRP and SAA, respectively) were tested with ten strips of the same batch to calculate the intra-coefficient of variation (intra-CV), and 3 batches were applied for the inter-coefficient of variation (inter-CV).

The blank control was tested for 20 times. The limit of blank (LOB) and the limit of detection (LOD) were calculated according to equations (2) and (3) in the Clinical Laboratory Standards Institute Guideline EP17-A2 [39].

$$LOB = Mean_{blank} + 1.645 \times SD \quad (2)$$

$$LOD = Mean_{blank} + 3 \times SD \quad (3)$$

Where $Mean_{blank}$ represented the average of the blank control and SD represented the standard deviation of the blank control within three times.

The PCT, CRP and SAA concentrations in 108 human plasma samples were detected by the triple LFIA method established in this paper for three times, respectively. Synchronously, they were detected by commercially available CLIA method (Roche for PCT, Simens for CRP and SAA). Patient consents were not obtained as all personal identifiers and patient information were delinked from the specimens. The Bland-Altman method was used for statistical data analysis.

3. Results and discussions

3.1. Principle of the multiple triple LFIA strip

The specific detection processes were shown in Scheme 1. When the sample was added onto the sample pad, it rapidly drenched on the conjugate pad 1 and touched mAb1-B-SA-PE. If target antigens of PCT, CRP, SAA existed in the sample, which would couple with the mAb1-B-SA-PE to form immuno-complexes respectively. Subsequently, these immune-complexes and PE could flow towards absorption pad and were captured by mAb2 on the T1, T2, and T3, respectively, but PE was captured by anti-PE mAb on the C line. There was a positive correlation between FI ratios of T1/C, T2/C and T3/C with relative to the antigen concentrations of PCT, CRP and SAA, and so three antigen concentrations in the sample could be quantified based on the FI ratios.

PE was a promising fluorescent material with quantifiable fluorescence intensity, narrow emission spectrum, long stokes shift (520/570 nm, Fig. S1), good biocompatibility and high solubility [33,35], which could eliminate auto-fluorescence interference from plasma biomolecules, such as pyridoxine, bilirubin, collagen and NAD(P)H (300–450 nm) [40], leading to reducing the impact of background noise. As a fluorescent probe, PE had been used in the detections of bovine plasma progesterone [41], nucleic acid [42] and human hCG [43] by LFIA. In the paper, we propose a novel triple LFIA strip with biotin-streptavidin-phycoerythrin (B-SA-PE) signal amplification system through the one-step self-assembly of biotin-labeled monoclonal antibodies (mAb-B) and SA-PE to rapidly form the mAb-B-SA-PE complex for the quantitative and simultaneous detection of inflammatory biomarkers (PCT, CRP and SAA) in serum samples.

The triple LFIA for simultaneous detection of PCT, CRP and SAA featured four particular advantages: 1) a uniform control line could avoid or reduce errors caused by testing for many strips; 2) the same fluorescent chromophore (PE) could facilitate the construction of detection devices and reduces instrument error; 3) three detection lines could reduce interference between each other; 4) an anti-PE monoclonal antibody was immobilized on NC membrane as control line, not conventional goat anti-mouse IgG, which minimized the interference of analytes and improved the accuracy. As expected, four lines (T1, T2, T3 and C) could be seen in positive samples (Fig. S2, lanes **1**–**5**) and one line in negative sample (Fig. S2, lane N) under UV light in the triple LFIA strips (Fig. S2). And under natural light, all the lines couldn't be seen by naked eyes.

3.2. Obtaining and characterization of anti-PE mAb

In order to minimize the error of difference between the sample and test strip, the T/C FI ratio was used to analyze sample quantitatively. Most LFIAs always immobilized the goat anti-mouse IgG on NC membrane to capture the excess antibody-conjugated fluorescence complex at the control line. However, the FI of the control line would be changed with the concentration of tested analytes which would result in serious cross interference in multiplex detection. To reduce the interference, a monoclonal antibody (anti-PE mAb) just reacted with PE, but not with SA-PE in the developed triple LFIA method. The anti-PE mAb was immobilized on NC membrane as capture antibody of a control line to capture PE. Thus, the FI of control line changed only with the different sample volume and viscosity, but not with concentration of the test analyte.

A number of successful fusions were achieved using mouse spleen cells which were immunized with purified PE protein. Approximately

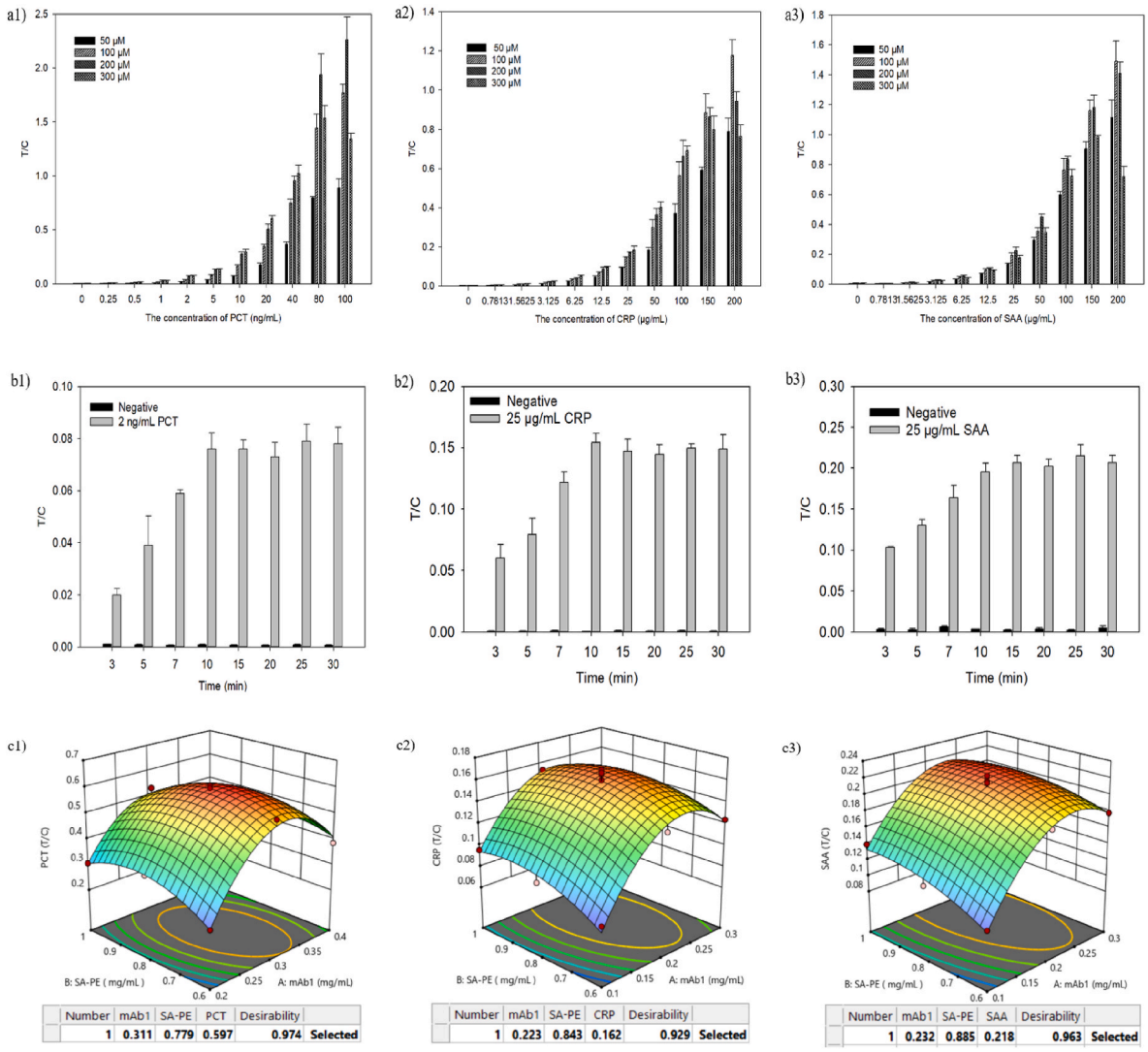


Fig. 2. The characters of mAb1-B-SA-PE self-assembly. (a1-a3) The effect of NHS-LC-Biotin content with mAb1 on self-assembly. (b1-b3) Effects of reaction time on self-assembly. The T/C ratio always increased with prolonging incubation time, and the reaction reached to the steady-state signal after 10 min (c1-c3) RSM patterns for mAb1-B and SA-PE self-assembly.

75% of fusion rate (93 hybridoma cultures) was achieved in seeded wells. Sixteen hybridomas produced antibodies against PE detected by indirect ELISA. Five mAbs (2D5, 3E10, 3G2, 4A9 and 5H7) were eventually isolated and expanded for further characterization study.

According to the indirect ELISA results, all mAbs could react positively with PE, and three mAbs (2D5, 3E10, and 4A9) showed positive reactions with SA-PE, and 3G2 was stronger reaction than 5H7. Therefore, 3G2 was selected for the following test. As seen in Fig. 1a, 3G2 was of subclass IgG1. 3G2 protein was extracted and identified by SDS-PAGE gel electrophoresis (Fig. 1b) and the molecular weight of the heavy chain was about 55 kDa and the light chain was about 25 kDa.

The affinity of anti-PE mAb was measured by non-competitive ELISA method. At the gradient of coating PE concentrations (0.5, 1, 2, 4 μg/mL), the 50% ODmax Anti-PE mAb concentrations were 50, 50, 25 and 12.5 μg/L, respectively (Fig. 1c). Anti-PE mAb (1 μg/L) was about 6.70 $\times 10^{-12}$ mol/L, and the affinity was 6.95 $\times 10^{-9}$ L/mol. Therefore, a monoclonal antibody cell was successfully obtained with high affinity and strong specificity only reacting with PE, which laid the foundation for the subsequent study in the triple LFIA.

3.3. Optimization of self-assembly of mAb1-B-SA-PE complex

The concentrations of PCT, CRP and SAA in human plasma were different by three orders of magnitude, which was a high challenge for the simultaneous detection [28,30]. PE was a promising fluorescent material with more than 1000 fold signal change [43], which characteristic was completely consistent with the determination of such a large range of concentrations in the system. Here we made use of 50-fold diluted samples compatible for the sensitivity of PCT, CRP and SAA.

The performances of NHS-LC-biotin and mAb1 were the main factors affecting the binding efficiency between mAb1-B-SA-PE complex and the analyte in plasma sample because the number of primary amino groups (-NH_2) in mAb1 was stationary, and too much or too little labeled ones would influence the fluorescence intensity. As shown in Fig. 2 a1-a3, upon increasing the NHS-LC-Biotin concentrations, the FIs gradually reached the maximum and then decreased, which suggested a saturated process of mAb1-B to SA-PE through the binding of biotin to streptavidin. After reaching the maximum, some binding sites of mAb1 to antigens (PCT, CRP and SAA) were nonspecifically occupied/covered by the SA-PE, which result in a decreased FIs. The optimal contents of NHS-LC-Biotin for the preparation of mAb1-B were 200 μM for PCT, 100 μM for CRP and SAA, respectively.

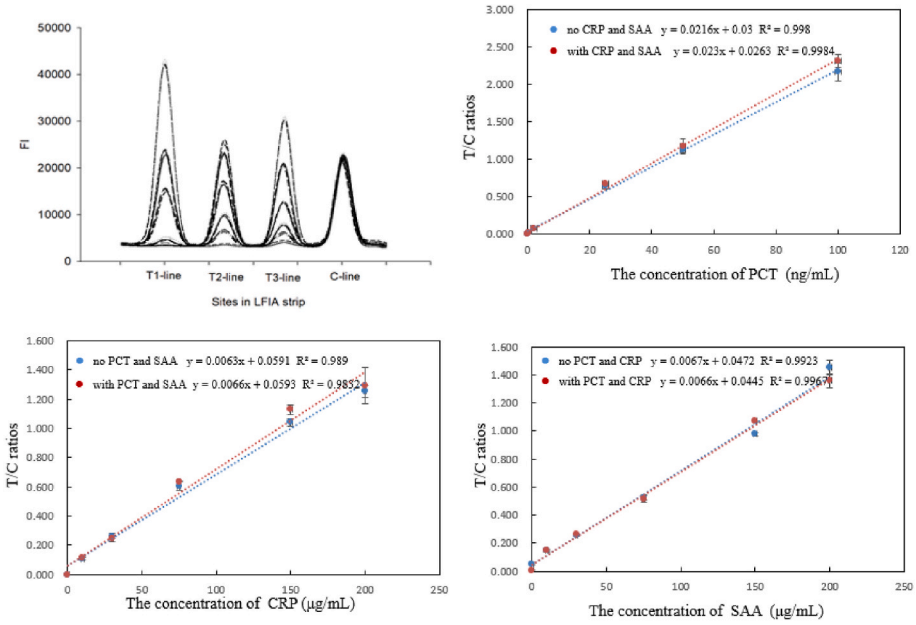


Fig. 3. Method validation. (a) Fluorescence spectra of captured mAb1-B-SA-PE in triple FLIA test. (b–d) The comparison of single and triple measurements in PCT, CRP and SAA.

The T/C ratio always increased with prolonging incubation time, and the reaction reached to the steady-state signal after 10 min (Fig. 2 b1-b3). Therefore, 10 min was select as optimum time in the immunoassay.

The contents of mAb1-B and SA-PE were optimized by the response surface methodology (RSM) to get the best sensitivity. After calculation, the optimal contents of mAb1-B and SA-PE were 0.311 and 0.799 mg/mL for PCT, 0.223 and 0.843 mg/mL for CRP, 0.232 and 0.885 mg/mL for SAA, respectively (Fig. 2 c1-c3).

3.4. Method validation

The standard curves of PCT, CRP and SAA were established based on three sets of serial concentrations of PCT, CRP and SAA standard antigens individually. Compared to T/C ratios of the same serial concentrations of triple (PCT, CRP and SAA) mixed standard antigens, the FI values of C lines kept constants as the concentrations of PCT, CRP and SAA increased (Fig. 3 a). As shown in Fig. 3 b-d, the calibration curves of the three biomarkers in the individual tests were plotted in blue dotted lines, and those in the triple tests were plotted in red dotted lines. There were no significant differences between the individually T/C ratios of PCT, CRP and SAA and the mixture of the three biomarkers. With the individual tests, the obtained calibration curves were $y = 0.0216x + 0.03$ ($R^2 = 0.998$) for PCT, $y = 0.0063x + 0.0591$ ($R^2 = 0.989$) for CRP and $y = 0.0067x + 0.0472$ ($R^2 = 0.9923$) for SAA. While in the triple tests, the calibration curves were $y = 0.023x + 0.0263$ ($R^2 = 0.9984$) for PCT, $y = 0.0066x + 0.0593$($R^2 = 0.9832$) for CRP and $y = 0.0066x + 0.0445$ ($R^2 = 0.9967$) for SAA. In other word, the calibration curves of triple tests were linear fitted and a good linearity between FI and the biomarker concentration as those of single biomarker test, indicating that no significant interference was found for the biomarkers in the triple assay.

LOB refers to the highest measurement result observed in measuring a blank sample, while LOD refers to the lowest concentration of analytes that can be qualitatively detected from samples within a given significance level. They are an important indicator in clinical testing. Based on 20 times blank control test, the LOBs for PCT, CRP and SAA were 0.088 ng/mL, 0.316 μg/mL and 2.581 μg/mL, and LODs for PCT, CRP and SAA were 0.106 ng/mL, 0.345 μg/mL and 3.112 μg/mL, respectively. The low LOB and LOD values for the three biomarkers indicated that the triple LFIA strip could meet the clinical requirements of the rapid and sensitive detection of inflammatory biomarkers. Meanwhile, the linearity ranges were 0.106–100 ng/mL for PCT, 0.345–200 μg/mL for CRP and 3.112–200 μg/mL for SAA, respectively. Additionally, the analytical performances of PCT, CRP and SAA were also compared to the results previously reported in Table 1 with single or two inflammatory biomarkers due to the lack of simultaneous detection of PCT, CRP and SAA platform. However, compared to these methods, the triple FLIA featured relatively bigger linear range, the shorter detection time and the lower

Table 1
Analytical performances in the LFIA platforms.

Label	Analyte	LOD	Detection time	Linear range	References
Au nanoparticles	CRP	0.001 μg/mL	15 min	0.1–5 μg/mL	[17]
Europium nanoparticles	PCT	0.5 ng/dL	20 min	0.5–100 ng/mL	[16]
	CRP	0.05 mg/dL		0.05–10 mg/dL	
Nanoparticle	PCT	0.12 ng/mL	35 min	0.12–11.827 ng/mL	[28]
	CRP	0.24 μg/mL		0.24–129.781 μg/mL	
Fe_3O_4@Au nanoparticle	CRP	0.01 ng/mL	30 min	0.01–500 ng/mL	[29]
	SAA	0.1 ng/mL		0.1–500 ng/mL	
Dual-QDs	CRP	6.37 ng/mL	30 min	10 -1000 ng/mL	[32]
	SAA	2.39 ng/mL		10 -1000 ng/mL	
phycoerythrin	PCT	0.106 ng/mL	10 min	0.106–100 ng/mL	This work
	CRP	0.345 μg/mL		0.345–200 μg/mL	
	SAA	3.112 μg/mL		3.112–200 μg/mL	

Table 2
Intra-assay and inter-assay precision of the triple-LFIA in PCT, CRP and SAA detection.

Index	Concentration	Intra-Assay Precision (n = 10)		Intra-Assay Precision (n = 3)	
		Mean ± SD	CV (%)	Mean ± SD	CV (%)
PCT (ng/mL)	0.50	0.49 ± 0.04	7.10	0.51 ± 0.04	8.02
	5.00	4.83 ± 0.35	7.34	4.99 ± 0.40	7.91
	50.00	59.19 ± 3.10	5.24	60.68 ± 4.32	7.12
CRP (μg/mL)	5.00	10.01 ± 0.69	6.86	10.06 ± 0.62	6.11
	50.00	50.61 ± 4.55	8.99	50.72 ± 4.55	8.96
	150.00	156.86 ± 7.27	4.63	153.46 ± 8.75	5.70
SAA (μg/mL)	10.00	9.99 ± 0.59	5.94	10.16 ± 0.66	6.46
	50.00	49.16 ± 3.53	7.19	51.24 ± 4.71	9.19
	150.00	152.76 ± 7.91	5.18	153.19 ± 8.31	5.42

LOD.

Intra-assay and inter-assay precisions were used to evaluate the reproducibility for PCT, CRP and SAA within three concentration levels. As shown in Table 2, the calculated intra-assay and inter-assay CV were lower than 10%, indicating a high precision of triple LFIA. Thus, the triple LFIA was proposed for quantitive analysis in on-site tests in clinical.

3.5. Application in clinical samples

A total of 108 human plasma samples was used to evaluate the practical application of triple LFIA. The measured results of PCT, CRP and SAA from triple LFIA were shown in Fig. 4a–c compared with corresponding ones measured by commercially available CLIA method. The Bland-Altman plots between the two methods were performed as shown in Fig. 4d–f, and the mean relative differences (95% limits of agreement) were 2.8%, 0.9% and 5.6%, revealing that there was no significant bias between those two methods. The correlation coefficients (R^2) for the Passing-Bablok regression analysis were 0.989 for PCT, 0.987 for CRP and 0.988 for SAA, respectively, indicating a good linear relationship between the two methods. These results meant that the triple LFIA method for PCT, CRP and SAA had a good performance compared with other commercialized methods. Combining the other significant advantages in rapidity, simple operation, low cost, the triple LFIA, therefore, showed a potential clinical application for the simultaneous quantitative detection of PCT, CRP and SAA in patient plasma samples.

4. Conclusions

In summary, triple LFIA strategy was developed for the simultaneous detection of PCT, CRP and SAA. Compared to the commercial CLIA method, triple LFIA featured a higher sensitivity and wider dynamic range in analyte concentrations within only 10 μL serum samples. Meanwhile, a portable fluorescence reader was used to record the fluorescence signals of three test lines and control line only through one excitation wavelength and to improve the accuracy with a lower LOD. The results suggested that the rapid and accurate triple LFIA could increase the access to real-time monitoring on distinguishing inflammation, bacterial infection, or viral infection and to provide guidance for the use of antibiotics or other medicines in the therapy of infectious diseases.

CRediT authorship contribution statement

Xiao-Ming Wang: Methodology, Data Collection, Writing – original draft. **Shan Li:** Methodology, Writing – review & editing. **Lin-Hai Li:** Data Collection. **Jian-Xun Song:** Data Collection. **Yan-Hua Lu:** Data Collection, Validation. **Zhi-Wei Zhou:** Methodology. **Lei Zhang:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

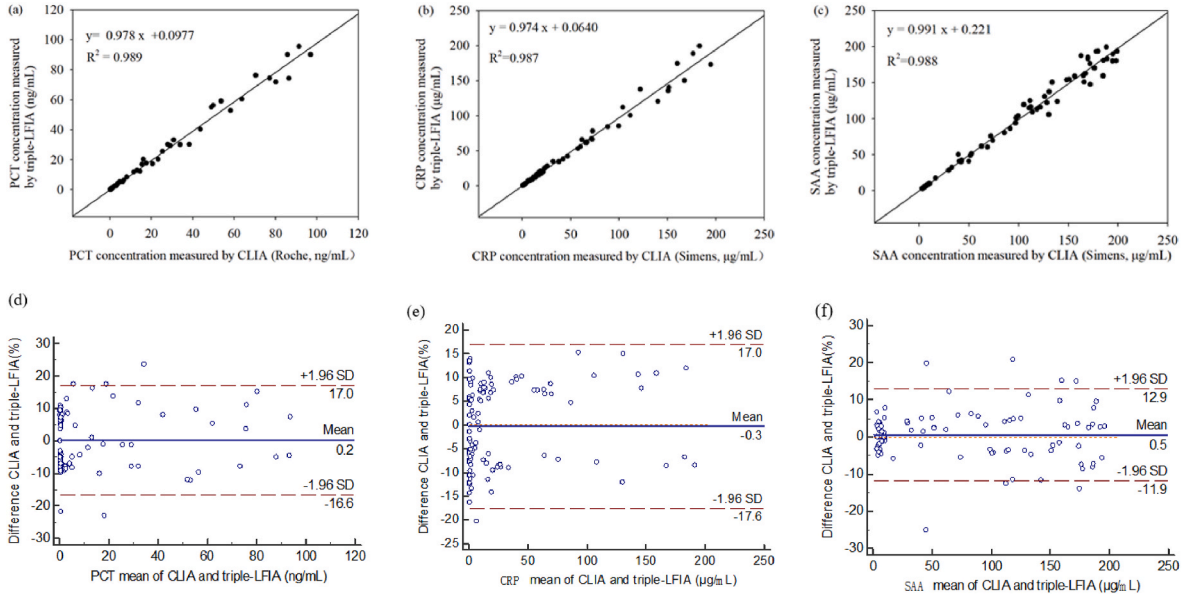


Fig. 4. The validation results from clinical samples for triple LFIA and CLIA. (a–c) The linear relationship between the results of triple LFIA and commercially used CLIA; (d–f) The Bland-Altman plot of mean relative differences for the triple LFIA and commercially used CLIA.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2022.114915.

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