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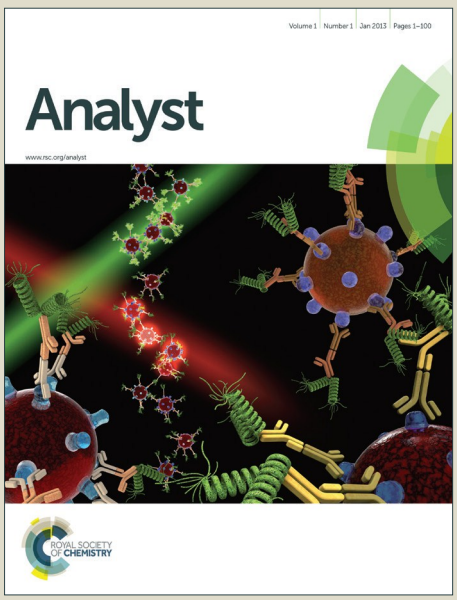

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Journal Name

ARTICLE

Combining Complement Fixation and Luminol Chemiluminescence for Ultrasensitive Detection of Avian Influenza A rH7N9

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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The complement fixation test (CFT) is a serological test that can be used to detect the presence of either specific antibody or antigen to diagnose infections. In a conventional CFT, the assay result is determined by observing the clarity of the reaction solution or sediment of red cells by eyes. Although the assay condition is thereafter simplified, the sensitivity of the assay would be sacrificed due to the limitation of bulk observation. Inspired by the forensic scientist to exam blood at a scene in criminal, we rationally argued that luminol chemiluminescence (CL) reaction could be applied in CFT to sense physiological complement-mediate haemolytic phenomena for sensitive protein detection. The combination of CFT and lumino CL system was demonstrated in detection of rH7N9, a recombinant avian influenza virus protein. The testing can be accomplished within 2.5 h and the linear detection range covers 0.25 fg/mL to 25 ng/mL. The feasibility of the CL based CFT in assaying real biopsy was successfully demonstrated by specifically detection rH7N9 and carcinoembryonic antigen (CEA) in human serum. This new type of protein detection approach inherits the beauty of complement-mediate assay, such as fast, no protein immobilization, blocking and washing. In addition, the participation of luminol CL enables quantitatively analyse the intensity of a hameolysis process, ameliorating the limitation of bulk observation in traditional CFT. It is anticipated the luminol CL-CFT assay would be particularly suitable for small molecules, toxic, short peptide investigation.

1. Introduction

In clinical practise and epidemiology, serological techniques play a critical role in various aspects of infection, vaccine development, and evaluation.[1] Serological tests, such as immunoassays, are performed in homogenous and heterogeneous formats. The key difference between these two types of assay is that homogenous assays do not require an additional physical separation step, and are generally simpler to perform with a shorter assay time. Most importantly, unlike heterogeneous sandwich assays, where an antigen (Ag) must possess two or more epitopes, homogeneous assays are appropriate for the analysis of small molecules, such as toxins and short peptides. The complement fixation test (CFT) is a homogenous serology test. The fundamental principle of this assay is that activation of the complement, a system of serum proteins, is initiated by complexes of Ags and IgG1, IgG2, IgG3, and IgM antibodies (Abs).[2, 3] When activation occurs on the cell surface, trans-membrane pores form, ultimately resulting in cell lysis.[4] This biochemical property has been utilized to develop the CFT for testing biomarkers of infectious and autoimmune diseases.[5-7] As illustrated in Figure 1, the CFT assay includes two systems: the sampler and indicator systems. The complement is added to the sampler system, which contains the unknown sample to be tested. Since the complement can be fixed/consumed by the Ab-Ag complex, this stage is also called the complex fixation stage. The indicator system (haemolytic system) contains Ab-coated erythrocytes (one type of Ag-Ab complex). If a target molecule exists in the sampler system, the recognition/binding of Ag and Ab leads to formation of a specific Ab-Ag complex that will fix/consume the complement (Fig. 1c). The residual complement can be further fixed/consumed by the Ab-

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

erythrocyte complex, leading to lysis of the erythrocytes (haemolysis) (Fig. 1a,b). Because the occurrence of haemolysis is dependent upon the non-activated complement remaining in the sampler system, the intensity of haemolysis indirectly represents the amount of Ab-Ag complex formed in the sampler system. The benefits of CFT compared to other serological tests, such as enzyme-linked immunosorbent assay (ELISA) and immunofluorescence (IF), is that there is no need for costly conjugates, and the assay time is shorter as it does not include protein immobilization, washing, and blocking steps.[6, 8, 9]

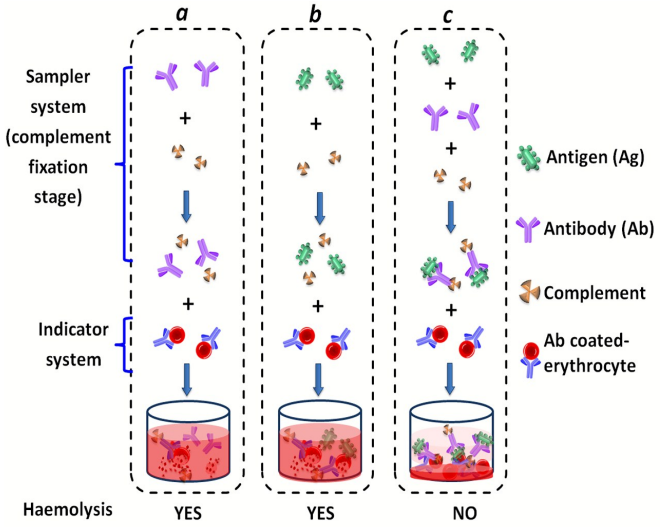

Fig. 1 Principle of the complement fixation test (CFT). There are two reaction systems in the CFT: Sampler system and Indicator system. ***a***. No target antigen (Ag) in the sample system. Free complement was activated by antibody (Ab)-coated erythrocytes, leading to haemolysis; ***b***. No target Ab in the sample system. Free complement was activated by Ab-coated erythrocytes, leading to haemolysis; ***c.*** There is target Ag-Ab in the sample system. The Ag-Ab complex was activated/consumed by the complement, resulting in no available complement for lysis of the erythrocytes in the indicator system.

In the conventional CFT, an assay result is normally determined by observing the clarity of the reaction solution or the sediment of red blood cells (RBCs) with the naked eye (Fig. 1). Although the assay condition has been simplified, its sensitivity may be compromised due to the limitation of bulk observation. Since haemoglobin leaks during haemolysis, we rationalized a sensitive analytical method to characterize the haemolysis process would enhance the benefit of CFT.

Luminol is a versatile chemical that can be oxidized to exhibit a striking blue glow, a phenomenon known as chemiluminescence (CL).[10] It is able to detect blood by reacting with the iron found in haemoglobin, a tetramer consisting of four polypeptide chains; the center of the tetramer contains four iron-containing molecules.[11] Previous studies have shown that the luminol CL system is catalysed by the iron in haemoglobin, allowing the sensitive detection of blood at crime scenes, as well as haemoglobin, myoglobin, and glycated haemoglobin.[12-15] Inspired by the forensic scientist to exam blood at a scene in criminal, we rationally argued that luminol CL reaction could be applied in CFT to sense physiological complement-mediate haemolytic phenomena for sensitive protein detection. In addition, the combination of CFT and lumino CL system was demonstrated in detection of rH7N9, a recombinant avian influenza virus protein. With the optimized usage of luminol/H_2O_2, the sensitivity of the CFT assay for rH7N9 detection was characterized. In addition, the assay is conducted with human serum sample to evaluate the feasibility for real biopsy analysis.

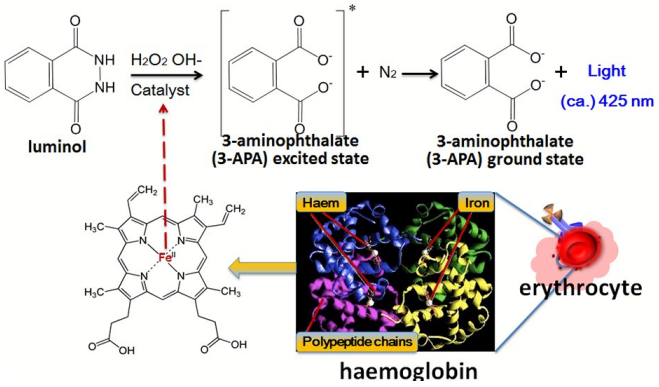

Fig. 2 Principle of luminol CL detection of haemolysis.

2. Experiment

2.1 Materials

Complement (purified from from guinea pig serum), erythrocyte (sheep red blood cell, SRBC) and its antibody (anti-SRBC antibody, haemolysin) were obtained from Nanfang reagent Inc., Shanghai, China. Carcinoembryonic antigen (CEA) and anti-CEA antibody was purchased from Sigma. Recombinant protein of avian influenza A (rH7N9) virus (A/Anhui/1/2013) and its specific antibody (anti-rH7N9 antibody) were ordered from Sino Biological Inc., China.

Commerical human serum was from Gibco Scientific Technology Co.LTD. Fresh, non-fasting serum samples were isolated from blood of a healthy volunteer. The blood was collected according to routine venipuncture protocols by hospitcal of Southwest Unviersity, and serum was separated from blood by centrifugation according to routine protocols. The samples were analyzed within 12 h of collection, therefore, these samples were considered to be "fresh."

Luminol was purchased from Aladin, China. It is dissolved in 0.1 M NaOH to a concentration of 1.77 mg/mL. Hydrogen peroxide (H_2O_2) was from Sinopharm Chemical Reagent Co. Ltd, China.

All other chemicals were bought from Sigma-Aldrich and used without further purification unless otherwise indicated. All solution was prepared with deionized (DI) water produced by PURELAB flex system, ELGA Corporation.

2.2 Testing of physiological complement-mediated haemolysis by luminol/H_2O_2 CL

To investigate if physiological complement-mediated cell lysis could be assayed by luminol chemistry, complement was mixed with Ab-coated erythrocytes to induce haemolysis. Sheep RBCs (erythrocyte) and the corresponding Ab (haemolysin) were used. The complement was purified from guinea pig serum (Nanfang Reagent). First, erythrocytes (4%) and haemolysin were mixed at a ratio of 1:125 (v/v) according to the manufacturer's instructions (Nanfang Reagent). A test group containing 10 µL of Ab-coated erythrocyte and 10 µL complement (25 unit/L) comprised the positive haemolytic group, and the negative

haemolytic group only included Ab-coated erythrocyte. After incubation at 37 °C for 1 h, the solution was mixed with 9 μL luminol (1.77 mg/mL in 0.1 M NaOH, Aladin, China) and 70 μL DI H_2O, then transferred into a micro-cuvette for photometer which was mounted on a fluorospectro-photometer (Shimadzu Co., Kyoto, Japan). Finally, 1 μL H_2O_2 (30%, Sinopharm Chemical Reagent Co. Ltd, China) was pipetted into the test samples, and the CL signal was then immediately measured on the fluorospectro-photometer. The scanning range covers wavelength of 350-600 nm. In addition, the morphology of the cells was examined under microscopy (Olympus IX71, Japan). All experiment was repeated three times in triplicates.

2.3 Complement fixation - luminol CL for protein detection

The CFT-luminol CL assay was divided into three steps. First, 10 μL of the sampler system containing 1.1 μg/mL anti-rH7N9 Ab, 25 units/mL complement, and serial concentrations of rH7N9 (2.5 fg/mL–2.5 μg/mL) was prepared and incubated at room temperature for 1 h. The mixture of antigen, antibody and complement setup the sampler system of CFT. Next, 10 μL of Ab-coated erythrocytes (1:125 ratio) was added to the sampler system, and incubated for another 1 h. Then, the 9 μL luminol (1.77 mg/mL) and 70 μL DI H_2O were pipetted into the complement fixation system and the solution was transferred into into a micro-cuvette. Finally, 1 μL H_2O_2 (30%) was pipetted into the cuvette and the CL intensity was immediately scanned using a fluorospectrophotometer. All experiments were repeated five times independently.

2.4 Testing real serological samples by CFT-luminol CL

To test the feasibility of the CFT-luminol CL for testing actual biological samples, commercialized human serum and serum isolated from a healthy volunteer were used to prepare the sample. Fresh, non-fasting serum samples were isolated from blood of a healthy volunteer. The blood was collected according to routine venepuncture protocols by hospital of Southwest University, and serum was separated from blood by centrifugation according to routine protocols. Serum samples were heat-inactivated to destroy self-containing complement prior to performing antibody assays. In brief, the serum samples were placed in water bath (60°C) for 3 minutes. The heat-inactivated serum was then centrifuged at 800 rpm for 5 minutes. Then, rH7N9 and tumor biomarker CEA protein were diluted with 10% serum to prepare the sampler system. To test the specificity of the CFT-luminol CL, CEA and rH7N9 proteins were tested. In detail, CEA or rH7N9 (2 ng/mL) was mixed with anti-CEA (1.3 μg/mL) Ab or anti-rH7N9 Ab (1.1 μg/mL) and complement for 1 h at 37°C. Then 10 μL of Ab-coated erythrocytes (1:125 ratio) was added to the sampler system, and incubated for another 1 h. With the adding of 9 μL luminol (1.77 mg/mL) and 1 μL H_2O_2 (30%), the CL intensity was immediately scanned using a fluorospectrophotometer. All experiments were repeated five times independently.

3. Results and Discussion

3.1 Physiological complement-mediated haemolysis can be characterized by luminol/H_2O_2 CL

In previous studies[12-14], to measure haemoglobin, harsh conditions were applied to destroy/lyse cells, or pure haemoglobin was used. To determine if physiological complement-mediated haemolysis can be characterized by the luminol/H_2O_2 reaction, four groups of samples were tested. In the reaction condition where Ab-coated erythrocytes were mixed with complement and luminol/H_2O_2 (positive haemolytic group), significantly fewer cells were observed (Fig. 3A) because the complement was fixed by the Ab coating the erythrocytes, leading to a cell lysis. The hemoglobin leaking from the lysed RBCs would catalyze the luminol CL reaction (Figure 2). Measurement of the CL signal showed that haemolysis corresponded with a sharply increased CL signal, indicating that physiological complement-mediated haemolysis can trigger the luminol CL reaction (Fig. 3B, curve **a**). In addition, no CL signal was seen from the mixture of RBCs and luminol/H_2O_2 (Fig. 3B, curves **b–d**). The results confirm that complement-mediated cell lysis can be assayed using the luminol CL reaction.

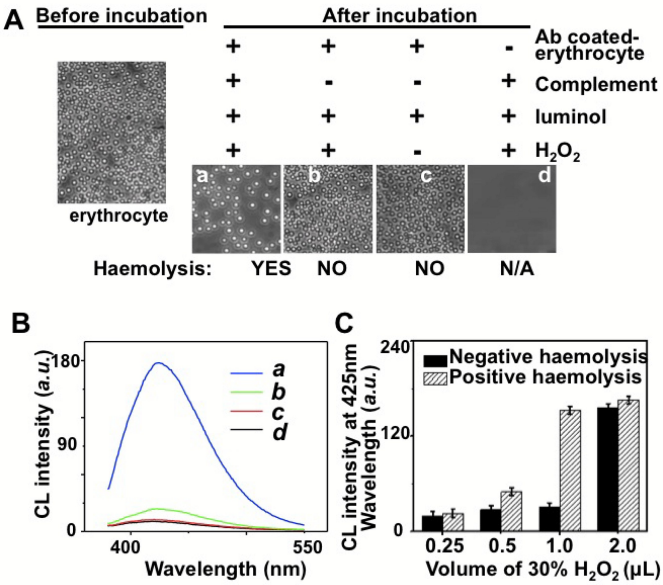

Fig. 3 The luminol CL signal specifically characterizes physiological complement-mediated haemolysis. (A) Microscopy image of erythrocyte cells under different conditions; (B) CL signal of erythrocytes under different conditions: ***a***. positive haemolytic group: Ab--coated erythrocytes + complement + luminol/H_2O_2; ***b***. negative haemolytic group: Ab-coated erythrocytes + luminol/H_2O_2; ***c***. Ab-coated erythrocytes + luminol; ***d***. complement + luminol/H_2O_2; (C) Optimization of H_2O_2 dosage in the luminol CL system. CL: chemiluminescence

It was reported that the haemoglobin did not react with luminol directly but were thought to form complexes with hydrogen peroxide, which can oxidize luminol chemiluminescently[15]. Hydrogen peroxide's dosage in the chemiluminescence system affects the signal intensity, while it would also potentially impair the integrity of the cell membrane, leading to nonspecific leakage of haemoglobin. Thus, different amount of H_2O_2 was added into the positive and negative groups to find the optimized condition for a better signal to noise ratio. The peak intensity at 425nm wavelength was compared. Figure 3C shows that mixing 1 μL

H_2O_2 (30%) with 9 μL luminol (1.77 mg/mL) can give a highest luminescence signal from positive haemolysis group while minimize the signal from negative group. This results indicate that optimized hydrogen peroxide can well participated in conversion of luminol to 3-aminophthalate (3-APA) excited state to produce luminescence signal (Figure 2), while avoiding the false positive signal by keeping the integrity of the cell.

3.2 Combination of complement fixation and luminol CL can achieve a sensitive protein testing

With the optimized luminol/H_2O_2 CL system, the complement fixation-based immunoassay was established to detect the recombinant subunit of Avian influenza A (A/Anhui/1/2013, rH7N9). H7N9 is a subtype of influenza virus that has been detected in birds. Outbreaks of human infection with the H7N9 virus occurred in China in the spring of 2013.[16-18] The first 3 days of an epidemic is the critical period when authorities need to take the appropriate action through internet-based surveillance to monitor and control the epidemic.[19] Established H7N9 detection methods, such as ELISA and virus isolation have the disadvantages of being multiple-steps procedures, with long assay times and high costs.[20-22] Thus, a fast, sensitive assay with a simple procedure is highly desired to study this virus. In this study, rH7N9 and its corresponding Ab were obtained from Sino Biological Inc. (Beijing, China). Figure 4A shows the CL intensity spectra measured with different concentrations of rH7N9. The positive haemolysis control (complement, Ab-coated erythrocyte, and luminol/H_2O_2) was characterized by the highest CL peak (*ca.* 425 nm) intensity (Fig. 4A, curve **a**), whereas the negative haemolysis control group containing Ab-coated erythrocytes and luminol/H_2O_2 had a low CL signal (Fig. 4A, curve **k**). Because the complement was fixed by recognition/binding of anti-rH7N9 Ab and rH7N9 Ag, less complement was available to react with Ab-coated erythrocytes, leading to a decreased CL signal (Fig. 4A, curves **b–j**). The protein concentration in the sample was calculated by changes in the intensity of complement-induced luminol CL (ΔCL %) as follows:

$$\Delta CL\% = [(CL_{haemolytic\ control} - CL_{sample})/\ CL_{haemolytic\ control}] \times 100\% \quad (1)$$

where $CL_{haemolytic\ control}$ is the CL signal of the haemolytic positive control and CL_{sample} is the CL signal measured from samples containing different concentrations of rH7N9. A higher ΔCL% indicates a higher concentration of rH7N9 in the mixture. The calibration curve of rH7N9 concentration versus ΔCL% calculated from three independent tests is shown in Figure 4B. The linear range covers 0.25 fg/mL–25 ng/mL (y=34.08496+7.390*log[Con.rH7N9], R^2=0.988) with a detection limit of 0.14 fg/mL based on noise to ratio of 3. The entire assay time was less than 2.5 h. Comparing with the existing analytical methods for detection of avian influenza A antigen (Table 1), the combination of complement-fixation and luminol chemiluminescence achieves a wider detection range and lower detection limit. More importantly, the assay was simple, as it did not involve protein immobilizaiton, washing, blocking, separation steps, and enzyme/fluorescent labeling.

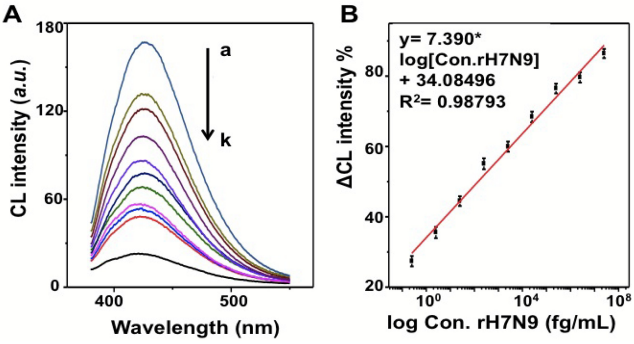

Fig. 4 The analytical performance of combining the CFT and luminol CL for detection of the recombinant H7N9 viral protein. **A**) Luminol CL signal vs. different concentrations of rH7N9 protein. **curve: a.** positive haemolysis control, **b.** rH7N9 0.25 fg/mL, **c.** rH7N9 2.5 fg/mL, **d.** rH7N9 25 fg/mL, **e.** rH7N9 250 fg/mL, **f.** rH7N9 2.5 pg/mL, **g.** rH7N9 25 pg/mL, **h.** rH7N9 250 pg/mL, **i.** rH7N9 2.5 ng/mL, **j.** rH7N9 25 ng/mL, **k.** negative haemolysis control; B) Calibration of changes of CL intensity *vs.* rH7N9 concentration.

Table 1 Analytical Performance of different methods for detecting avian influenza virus antigen

Method	Linear range ng/mL	Detection limit ng/mL	Exempt from		Label-free	Ref
			Protein immobilization	Wash Process		
CFT-based CL	$2.5X10^{-7}$-2.5	$1.4x10^{-7}$	Yes	Yes	Yes	*a*
microfluidic EC immunosensor	0.001-10	0.001	No	No	No	23
EIS	-	$5X10^{3}$	No	No	Yes	24
Indirect fluorescence	0.27-12	0.09	No	Yes	No	20
sFLISA	8-510	0.15	No	No	No	25
Resonance light scattering	0.5-50	0.15	No	No	No	26
NPs-based IA	-	10	No	No	No	27
DAS-ELISA	-	2.5	No	No	No	28

a. this work

CFT: complement fixation test**; CL:** Chemiluminescence**; EC:** electrochemical; **EIS**: electrochemical impedance spectroscopy; **FIA**: fluoroimmunoassay; **sFLISA:** sandwich fluorescent-linked immunosorbent assay; **DAS**: double antibody sandwich; **ELISA**: enzyme-linked immunosorbent assay;

3.3 Complement fixation-luminol CL combination is feasible for real serological sample testing

To explore the feasibility of the CFT-CL assay for testing an actual biopsy sample, the sampler system was prepared with commercialized human serum and serum provided from a healthy volunteer according to hospital regulations. Following the same experimental protocol, carcinoembryonic antigen (CEA) and rH7N9 protein (2 ng/mL) were spiked into the human serum to setup the sampler system. Experimentally, six samples were measured in the sampler system: 1) positive haemolysis control (no Ag or Ab); 2) CEA Ag + anti-CEA Ab; 3) rH7N9 + anti-rH7N9 Ab; 4) rH7N9 + anti-CEA Ab; 5) CEA + anti-

rH7N9 Ab; and 6) negative haemolysis control (rH7N9 Ag + anti-rH7N9 Ab). Complement (25 unit/L) was added into each sampler groups except the negative haemolysis control. Since there was no Ag or Ab in group 1, the complement can react with Ab-coated erythrocytes, leading to cell lysis (positive haemolysis). However, without complement in sampler system of group 6, no cells can be lysed by the complement (negative haemolysis control). The results show that the luminol CL curves of the mispaired Ag-Ab (rH7N9 + anti-CEA Ab, CEA + anti-rH7N9 Ab) were nearly identical to the positive haemolysis control, indicating that no Ag-Ab complex formed in the sampler system to fix the complement. On the other hand, recognition of the rH7N9/anti-rH7N9 Ab and CEA/anti-CEA Ab led to a significantly decreased CL signal (Fig. 5A). Because the spiked paired CEA Ag-CEA Ab and rH7N9 Ag-rH7N9 Ab fixed the complement in the sampler system, fewer complement can react with the Ab-coated erythrocytes, as represented by larger CL changes (ΔCL%). The specificity of the test was determined by the biochemical property of the complement that could only be activated by the paired Ag-Ab complex in the sampler system.[2, 3] In addition, the ΔCL% of paired Ab-Ag from reaction systems of commercialized human serum and fresh serum isolated from health donor are slightly smaller than the value from DI water group, but significantly higher than corresponding non-paired Ag-Ab control groups (Figure 5B). These results demonstrate that combining the CFT and luminol CL can work well for protein detection, thereby showing great potential for evaluating serological samples.

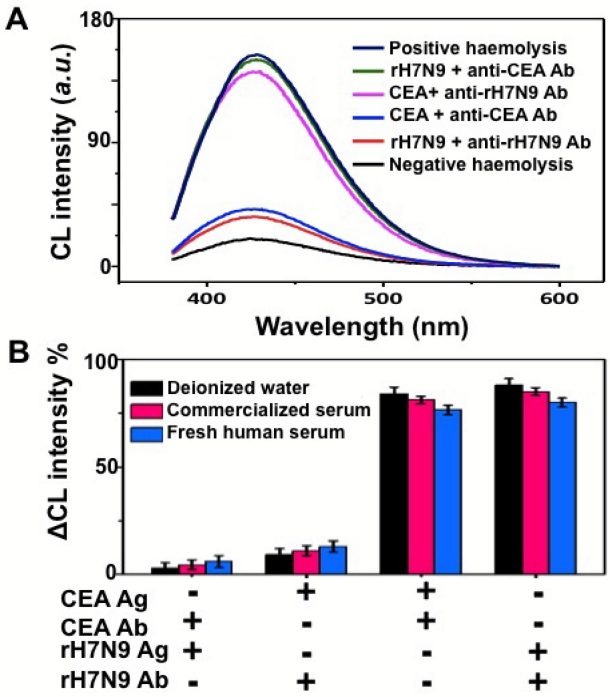

Fig. 5 Specificity of the CFT luminol CL test. **A**) Luminol CL signal *vs.* different pairs of Ab-Ag in sampler system**.** B) Histogram of changes of CL intensity *vs.* different pairs of Ag-Ab in the sampler system. CEA: carcinoembryonic antigen, Ab: antibody, Ag: antigen

It was very significant to see that with the combination of CFT and luminol CL, the detection limit of recombinant viral protein increased to 0.25 fg/mL, which is highly comparable to other complicated and time-consuming methods.[20, 22] This ultrahigh sensitivity could be due to the following: 1) the homogenous assay format avoids damage to the structure and function of the Ab and Ab, allowing formation of the Ag-Ab complex; 2) The CFT assay is sensitive in that only one Ab-Ag complex initiates the cascade of complement activation and leads to haemolysis; and 3) ultrasensitive haemoglobin catalyses luminol CL, and the detection limit of luminol CL for haemoglobin is up to 40 pM[15]. Since the average haemoglobin per erythrocytes is 1.6–1.9 pM[29], the luminol CL reaction can detect protein from 20 lysed cells. Considering there is about 3–5 million RBCs per mL, the ability to detect only 20 cells is very impressive. Thus, the combination of the luminol CL system and the CFT enhances the sensitivity of the assay for protein detection. This new type of protein detection approach maintains the benefits of the complement-mediated assay, such as the speed, lack of protein immobilization, blocking, washing steps, and absence of enzyme/fluorescent conjugates. In addition, the addition of luminol CL enables the quantitative analysis of the haemolysis process, ameliorating the limitation of bulk observation associated with the traditional CFT.

Conclusion

In conclusion, for the first time, the luminol CL system was applied in combination with the CFT for the ultrasensitive detection of proteins. Physiological complement-mediated cell lysis and haemoglobin leaking can trigger catalysis of luminol to emit light. The combination of CFT and the luminol CL system was demonstrated for the detection of rH7N9, an avian influenza virus. The entire assay was completed within 2.5 h. The range of linear detection was 0.25 fg/mL–25 ng/mL, and the limit of detection was 0.14 fg/mL. The protocol did not include time-consuming washing or blocking steps, or expensive chemical conjugates, highlighting its advantages over heterogeneous immunoassays such as ELISA or fluorescent immunoassay. Most importantly, all of the biochemical reactions involved in this new type of CFT based Ag/Ab detection are well characterized, and can be performed with relatively simple instrumentation. We believe that the luminol CL-CFT assay will be particularly valuable for detecting small molecules, toxins, and short peptides.

Acknowledgements

This work is financially supported by the National Science Foundation of China (No. 31200700， 21375108), Science Foundation of Chongqing (cstc2014jcyjA10070) and Fundamental Research Funds for the Central Universities (XDJK2015B020).

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