



## ORIGINAL ARTICLE

# Development of a C3c-based ELISA method for the determination of anti-complementary potency of *Bupleurum* polysaccharides



Mulu Wu<sup>a,1</sup>, Hong Li<sup>a,\*</sup>, Yunyi Zhang<sup>a</sup>, Daofeng Chen<sup>b</sup>

<sup>a</sup>Department of Pharmacology, School of Pharmacy, Fudan University, Shanghai 201203, China

<sup>b</sup>Department of Pharmacognosy, School of Pharmacy, Fudan University, Shanghai 201203, China

Received 7 November 2014; received in revised form 30 January 2015; accepted 26 February 2015

## KEY WORDS

ELISA;  
Complement C3c;  
Suramin;  
*Bupleurum smithii* var.  
*parvifolium*;  
*Bupleurum chinense*;  
Polysaccharides

**Abstract** Traditionally, determination of inhibitory potency of complement inhibitors is performed by the hemolytic assay. However, this assay is not applicable to the lectin pathway, thus impeding the understanding of complement inhibitors against the overall function of the complement system. The main objective of our study was to develop a specific enzyme-linked immunosorbent assay (ELISA) as an alternative method to assess the anti-complement activity, particularly against the lectin pathway. By using respective coating substrates against different activation pathways, followed by capturing the stable C3c fragments, our ELISA method can be used to screen complement inhibitors against the classical pathway and the lectin pathway. The inhibitory effect of suramin on the classical pathway, as measured by our hemolytic assay is consistent with previous reports. Further assessment of suramin and *Bupleurum* polysaccharides against the lectin pathway showed a good reproducibility of the method. Comparison of the lectin pathway IC<sub>50</sub> between *Bupleurum smithii* var. *parvifolium* polysaccharides (1.055 mg/mL) and *Bupleurum chinense* polysaccharides (0.98 mg/mL) showed that, similar to the classical and alternative pathway, these two *Bupleurum* polysaccharides had comparable anti-complementary properties against the

**Abbreviations:** Abs, antibodies; AP, alternative pathway; BCPs, *Bupleurum chinense* polysaccharides; BG, background value; BPs, *Bupleurum smithii* var. *parvifolium* polysaccharides; BSA, bovine serum albumin; CP, classical pathway; CV, coefficient of variation; DFC, drug-free control; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; LP, lectin pathway; LPS, lipopolysaccharide; MASP, MBL-associated serine proteases; MBL, mannose-binding lectin; OD, optical density; PBS-T-BSA, PBS containing 0.05% Tween-20 and 1% BSA; SRBC, sheep erythrocytes; VBS, Veronal buffer saline

\*Corresponding author. Tel.: +86 21 51980050.

E-mail address: [lxzhang@shmu.edu.cn](mailto:lxzhang@shmu.edu.cn) (Hong Li).

<sup>1</sup>Present address: Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117545, Singapore.

Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.

<http://dx.doi.org/10.1016/j.apsb.2015.02.004>

2211-3835 © 2015 Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

lectin pathway. The results demonstrate that the described ELISA assay can compensate for the shortcomings of the hemolytic assay in lectin pathway.

© 2015 Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 1. Introduction

As one of the major effector mechanisms of innate immunity, the complement system is involved in a broad range of physiological and pathological processes, such as clearance of immune complexes and chronic inflammation<sup>1</sup>. Over the past decades, researchers have shown that besides its obvious roles in host defense, the complement system also closely interacts with other cascade effector systems, for example, the coagulation system<sup>2</sup>. By participating in such diverse processes, the intricate complement system builds up a delicate balance that is tightly regulated. However, without proper regulation, these cascades can generate a lot of inflammatory factors, which could disrupt the homeostasis and ultimately contribute to various immune, inflammatory and neurodegenerative diseases<sup>3</sup>. Hence, in the past 10 years, there has been a growing concern with an effort to develop therapeutics, especially complement inhibitors that can block the complement cascade and thus prevent the hyperactivity of various complement components.

In order to discover complement inhibitors, more effective and efficient methods are required. The traditional hemolytic assay is the most commonly used assay to determine the activity of complement system *via* three pathways respectively, namely the classical pathway (CP), alternative pathway (AP) and lectin pathway (LP). Hemolysin sensitized sheep or rabbit erythrocytes are mixed with compounds to be tested. The more active the complement system, the more erythrocytes get lysed, *i.e.*, less serum is needed to lyse 50% of a fixed amount of erythrocytes.

Although the hemolytic assay is simple and straightforward, the shortcomings of the assay are also apparent. The LP is activated when mannose-binding lectin (MBL) binds to one of its carbohydrate ligands on the bacterial surfaces. The binding subsequently results in the association of two serine proteases: MASP-1 and MASP-2 (MBL-associated serine proteases). However, the MBL-MASP complex has a strong structural and functional similarity to the C1 complex, thus leading to the interference of the CP involved with the hemolytic MBL assay<sup>4</sup>. It has been shown that this interference can result in approximately 25% overestimation of MBL-mediated hemolysis or even false test results in patients with MBL-deficient genotypes<sup>5</sup>, unless certain antibodies, such as anti-C1q antibodies, are added into the system to inhibit the CP activation.

The clinical significance of the LP is increasingly noticeable for the past decades. Its importance is highlighted in anti-bacterial infection, especially in earlier years of life before adaptive system has been established<sup>6</sup>. Despite of its protective role, inappropriate activation of the LP could result in some life-threatening disease, such as ischemia-reperfusion injuries and kidney diseases<sup>7</sup>. Therefore, the defect of hemolytic assay in the LP limits the overall understanding of complement inhibitors, particularly against the LP. So far, many polysaccharides extracted from various plants, such as the *Bupleurum chinense* polysaccharides, have shown anti-

complementary features against the CP and the AP, but their efficacy in the LP is still uncovered due to this defect<sup>8</sup>. Besides, as the readout of the hemolytic assay is optical density (OD), controls are always needed to eliminate the baseline OD of colored compounds. In this case, hemolytic assay is not an ideal assay for drug various screening as it requires substantial amount of purified compounds.

Apart from the hemolytic assay, the complement function can also be evaluated by means of the ELISA technique *via* antibodies<sup>9–11</sup>. The sera investigated are incubated in microtiter plates coated with solid-phase complement activators. Human IgG or IgM, lipopolysaccharide (LPS) and mannan are used as activators respectively for CP, AP and LP. A specific antibody against the corresponding complement component (*e.g.*, C3/C4) is then used to detect the complement activation. Compared with hemolytic assay, the ELISA allows for detection of complement activation in all three pathways. Up to now, this method has only been employed to determine the complement activation or deficiency in biological samples from patients or model animals. Considering that the ELISA assay has several washing steps, it can eliminate the influence of compounds' color on the final readout. Additionally, the 96-well plate in which ELISA is carried out also meets the requirement for high throughput screening.

As mentioned above, a specific antibody is needed in the ELISA assay to capture a particular cleavage product to reflect the degree of complement activation. All three pathways, though activated by different substrates, converge at C3, resulting in the formation of the activation products, C3a, C3b, C5a and then ultimately the membrane attack complex (C5b-9)<sup>12</sup>. In contrast to C5a fragment, which is highly active with a half-life less than 5 min<sup>13</sup>, various C3 activation products are readily detectable as they remain for a few hours<sup>14</sup>. Recent findings indicate that the stable C3 cleavage product C3c is a valuable marker for inflammatory processes<sup>15</sup>. Based on this, we chose the C3c complement fragment as an indicator of complement activation.

In this paper, we developed a C3c-based ELISA method adapted from the protocol reported by Roos et al.<sup>4</sup> and examined if this modified assay could be used for the screening of complement inhibitors, especially for the determination of anti-complementary effects against the LP. Next, the described assay was verified by determining the inhibitory effect of the known anti-complement drugs suramin and *Bupleurum* polysaccharides against the CP and the LP.

## 2. Materials and methods

### 2.1. Animals

Male guinea pigs, 4 weeks old of (300±50) g body weight, were purchased from Slaccas-Shanghai Lab Animal Ltd. The guinea pigs were kept under specific pathogen free and normal housing

conditions with access to water and food. All experimental protocols described in this study were approved by the Animal Ethical Committee of School of Pharmacy, Fudan University.

## 2.2. Preparation of guinea pig serum

The guinea pigs were anesthetized and whole blood was collected from the femoral artery. After that, the blood was incubated at 4 °C for 1 h to allow clotting. 200 µL of Veronal buffer saline (VBS)-washed (145 mmol/L NaCl, 1.8 mmol/L sodium barbiturate, 2.8 mmol/L barbiturate acid) and packed sheep red blood cells were mixed with every 10 mL sera and spin for 10 min at 400 × *g*. The supernatant was collected and aliquots were stored at -80 °C for further use.

## 2.3. C3c based ELISA

In general, ELISA was performed using 96 well microtiter plates (Nunc) that were coated with 50 µL/well of different substrates diluted in coating buffer (100 mmol/L Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub>, pH 9.6). After overnight incubation at 4 °C, the coated wells were washed three times after which the nonspecific binding sites were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at 37 °C. All subsequent detection antibodies were diluted in PBS containing 0.05% Tween-20 and 1% BSA (PBS-T-BSA) unless otherwise stated, and each step was followed by washing three times using PBS-0.5% Tween-20. Subsequently, the guinea pig sera were incubated at indicated dilutions for 1 h at 37 °C. After washing, bound C3c was detected using diluted rabbit polyclonal anti-human C3c antibodies (Abs) (Shanghai Long Island Biotech. Co., Ltd., D-3004), followed by horseradish peroxidase (HRP)-conjugated anti-rabbit Abs (Shanghai Long Island Biotech Co., Ltd., P-0047) diluted in PBS-T-BSA, for 1 h at 37 °C respectively. Enzyme activity of HRP was assessed by addition of *o*-phenylenediamine and H<sub>2</sub>O<sub>2</sub>. After 15–30 min the reaction was stopped by addition of 50 µL of 1 mol/L H<sub>2</sub>SO<sub>4</sub> per well. OD was measured at 495 nm using a microtiter plate reader (Multiskan FC, Thermo Scientific). The complement components of human and guinea pig share structural similarity<sup>16</sup>, hence the anti-human C3c Abs used in our assay were able to capture the bound C3c fragments in the guinea pig serum. The coating concentration of IgM and LPS was determined by using rabbit polyclonal anti-human C3c Abs and HRP-conjugated anti-rabbit Abs at 1:1000. The optimization of dilutions for these two Abs was carried out on the LP.

For the detection of CP activity, Nunc plates were coated with human IgM at 5 µg/mL (Beijing Biosynthesis Biotechnology Co., Ltd., bs-0345P) overnight at 4 °C in coating buffer. Sera were incubated in serial dilutions in BVB++ (VBS containing 0.5 mmol/L MgCl<sub>2</sub>·6H<sub>2</sub>O, 1.5 mmol/L CaCl<sub>2</sub>, 0.05% Tween-20, 1% BSA, pH 7.5), for 1 h at 37 °C. For the LP, plates were coated with mannan at 100 µg/mL (Sigma, M7504). After washing, serial dilutions of sera in BVB++ buffer were added and incubated for 1 h at 37 °C. For AP, plates were coated with LPS at 10 µg/mL (Sigma, L3129). Sera were then incubated in GVB/Mg-EGTA buffer (VBS containing 10 mmol/L EGTA, 5 mmol/L MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.05% Tween-20, 0.1% gelatin, pH 7.5).

## 2.4. Anti-complement activity determination

After optimization of the different parameters, conventional complement inhibitors suramin (Sigma, S2671) and *Bupleurum*

polysaccharides were used for the validation and application of the assay. Crude polysaccharides isolated from *Bupleurum smithii* var. *parvifolium* (BPs, H2003121602) and *Bupleurum chinense* (BCPs, 20071015) were obtained from Prof. Daofeng Chen (Department of Pharmacognosy, School of Pharmacy, Fudan University, Shanghai, China). To determine the IC<sub>50</sub> values against the CP and LP, compounds were serially diluted and pre-incubated with guinea pig sera for 45 min at 37 °C. The compound/sera mixture was then added to the coated 96-well plates and processed as the ELISA method described above. A tube of compound-free BVB++ buffer mixed with sera was also introduced in the experiment as drug-free control (DFC). Background value (BG) is determined as the OD value of wells added heat-inactivated fetal bovine serum instead of guinea pig serum. All experiments were carried out in triplicates in 96-well plate. The percentage of inhibition is calculated according to the following equation:

$$\text{Inhibition percentage (\%)} = 100 - (OD_{\text{sample}} - OD_{\text{BG}}) / (OD_{\text{DFC}} - OD_{\text{BG}}) \times 100$$

The IC<sub>50</sub> values determined by our ELISA were compared with the results of the hemolytic assays published previously. The intra-assay precision was assessed by analyzing 3 replicates of each sample in a single run. The inter-assay precision was analyzed in triplicate in 3 separate run.

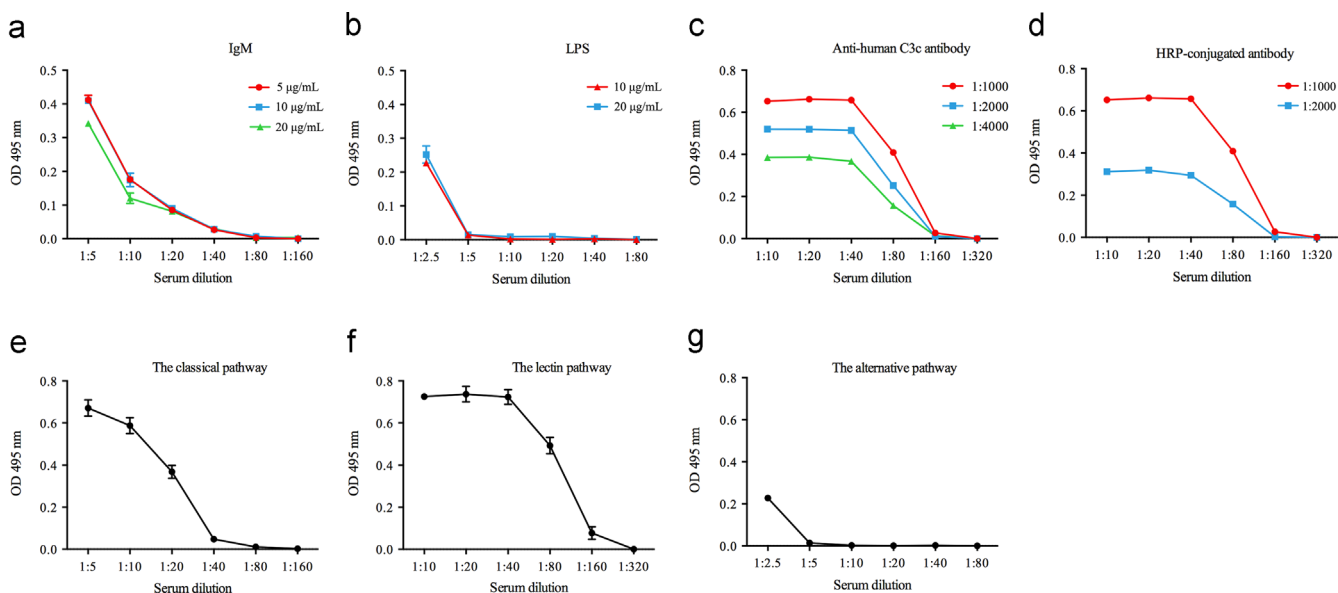
## 3. Results

### 3.1. Assay optimization

The assay used in this experiment was adapted from Roos et al.<sup>4</sup> and Trouw et al.<sup>17</sup>. Considering the difference in coating substrate and antibodies, various concentrations of all the reagents were tested in order to optimize the assay. Different dilutions of sera were used to represent varied complement level in the assay.

The mannan used for the LP was obtained from the same supplier (Sigma-Aldrich, M7504) as Roos et al.<sup>4</sup>, thus the coating concentration (100 µg/mL) was adopted from their work. For the CP and the AP, three concentrations of IgM (5, 10 and 20 µg/mL) and two concentrations of LPS (10 and 20 µg/mL) were tested. Our results showed that different concentrations of IgM had similar level of activation on the CP (Fig. 1a), suggesting that the binding site on the microtiter plate was already saturated at 5 µg/mL. Similarly, 20 µg/mL LPS had comparable results as 10 µg/mL in the AP (Fig. 1b). Therefore, in our assay, the coating concentration for IgM was chosen to be 5 µg/mL and 10 µg/mL for LPS.

The second step of this assay was to determine the concentrations of rabbit polyclonal anti-human C3c Abs and HRP-conjugated anti-rabbit Abs. These were carried out on the LP. For rabbit polyclonal anti-human C3c Abs, three dilutions (1:1000, 1:2000 and 1:4000) were tested, keeping the HRP-conjugated anti-rabbit Abs dilution constant at 1:1000 (Fig. 1c). To achieve high intensity of color, 1:1000 of anti-human C3c Abs was determined to be the best. Next, in order to decide the amount of HRP-conjugated anti-rabbit Abs needed in the assay, the HRP-conjugated anti-rabbit antibody dilutions were tested from 1:100 to 1:25,600 (data not shown) using IgG standard in the well-established IgG ELISA assay in our lab (previously described in Jiang et al.<sup>18</sup>). Dilutions of 1:800 and 1:1600 gave relatively higher OD values with a broader linear range. Based on this result, two dilutions of 1:1000 and 1:2000 were further verified using the ELISA method for the LP as described above. As a result, 1:1000



**Figure 1** Assay optimization. (a and b) Complement activation curve for different coating concentrations of (a) IgM and (b) LPS. (c and d) Complement activation curve for different (c) HRP-conjugated anti-rabbit antibody dilutions and (d) rabbit polyclonal anti-human C3c antibody dilutions. (e–g) Standard curves for (e) the classical pathways, (f) the lectin pathway and (g) the alternative pathway. Data (mean  $\pm$  SD) from triplicates are representative of three independent experiments.

dilution gave a better signal-noise ratio ( $>10$ ) and thus was selected as the optimum dilution used in the assay (Fig. 1d).

Our assay was set up to screen complement inhibitors. For this purpose, the optimum serum concentration should meet two requirements: 1) give a good signal to noise ratio; and 2) be reliable enough to determine that the decreased OD is due to real inhibitory effects and not false positive results caused by experimental errors, *i.e.*, the complement activation should not be too sensitive at the concentration of interest in the standard curve. For the CP, the guinea pig serum concentration was determined by varying the dilutions from 1:5 to 1:160. As can be seen in Fig. 1e, none of the dilutions presented good stability. The dilutions of 1:5 and 1:10 had relatively higher OD value and medium level of sensitivity compared with the dilution of 1:20. According to the 3Rs principle (replacement, reduction and refinement) for experiments, the dilution of 1:10 was chosen instead of 1:5 so as to consume less serum from guinea pigs. Similarly, a range of sera diluted from 1:10 to 1:320 was conducted for the LP (Fig. 1f). The dilution of 1:20 for the LP was selected also due to reduced guinea pig sera consumption and at this dilution, the assay demonstrated less variation.

However, for the AP (Fig. 1g), at high concentration of sera (1:2.5 dilution), the OD value was fairly low. All the sera including those which were more diluted, showed almost no activation at all, suggesting that this ELISA method might not be suitable for the AP. Since the inhibitory effects of complement inhibitors on both the CP and the AP can be studied *via* hemolytic assay, the following experiments were mainly focused on the validation and application of LP.

### 3.2. Assay validation and application

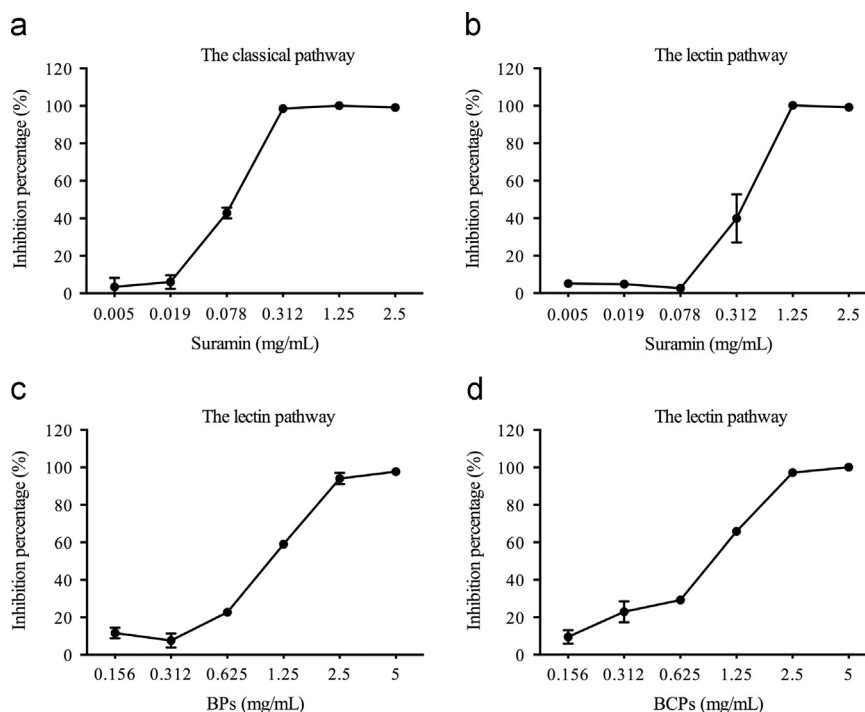
After determining the optimal conditions for all parameters, we moved on to evaluate the precision of our assay by studying the inhibitory activities of tested compounds. Due to the shortcoming of the hemolytic assay, there were no verified LP inhibitors available for us to use as positive control. Therefore, we checked

the reliability of the assay by measuring the  $IC_{50}$  of well-characterized complement inhibitor suramin on the CP and comparing it with the hemolytic assay. Suramin has been found to inhibit at least six different enzymes in the complement systems competitively, including C1, C2, C4 and serine proteases<sup>19</sup>. C2 and C4 play an important role in the CP, while serine proteases (MASPs) are one of the most important components in the LP. Suramin was tested starting from 2.5 mg/mL and then serially diluted until 0.005 mg/mL. Based on the equation generated from the inhibition potency curve obtained from the ELISA assay (Fig. 2a), we determined that the  $IC_{50}$  of suramin on the CP is  $0.089 \pm 0.011$  mg/mL, which is in agreement with literature reports of a  $CH_{50}$  around 0.1 mg/mL determined by hemolytic assay<sup>19</sup>. This indicates that our ELISA assay gives reliable results that are comparable to the results obtained by hemolytic assay.

Furthermore, we examined the reproducibility of the assay by subjecting two classes of known complement inhibitors to the LP: 1) suramin, as a representative for synthesized compounds; and 2) BPs, a well-studied anti-complementary compound in our lab, as a representative for colored natural products. The reproducibility of the assay was determined by analyzing 3 replicates in a single run for intra-assay precision and 3 independent replicates for inter-assay accuracy. Suramin was tested within the same range of concentrations as used for the CP and BPs were tested with final concentrations ranging from 0.156 to 5 mg/mL. Inhibitory effect curves are presented in Fig. 2b and c. Results showed that both suramin and BPs have anti-complement potency on the LP with a determined  $IC_{50}$  value of  $0.368 \pm 0.071$  mg/mL and  $1.057 \pm 0.003$  mg/mL respectively. For both intra-assay and inter-assay assessment of suramin and BPs, the obtained values of coefficient of variation (CV) are smaller than 30% (Table 1), and thus ascertain a good reproducibility of the assay.

In addition to BPs, another polysaccharides extracted from BCPs were also tested in our ELISA assay to study its inhibitory effect on the LP. Similar to BPs, BCPs were tested ranging from 0.156 to 5 mg/mL as shown in Fig. 2d. The determined  $IC_{50}$  value





**Figure 2** Assay validation and application with suramin, *Bupleurum smithii* var. *parvifolium* polysaccharides (BPs) and *Bupleurum chinense* polysaccharides (BCPs). (a) Inhibitory effect of suramin on the classical pathway. (b) Inhibitory effect of suramin on the lectin pathway. (c) Inhibitory effect of crude polysaccharides isolated from BPs on the lectin pathway. (d) Inhibitory effect of BCPs on the lectin pathway. Results are expressed as percent inhibition ( $n=3$ ). Mean  $\pm$  SD is shown in duplicates and data are representative of three independent experiments.

**Table 1** Determined  $IC_{50}$  values (mg/mL) and statistics data of suramin and BPs on the lectin pathway.

Sample	ELISA assay (Mean, mg/mL)		Standard deviation (SD)		Coefficient of variation (CV, %)	
	Inter-assay <sup>a</sup>	Intra-assay <sup>b</sup>	Inter-assay	Intra-assay	Inter-assay	Intra-assay
Suramin	0.368	0.318	0.071	0.028	19.21	8.81
BPs	1.057	1.055	0.215	0.169	20.37	16.02

<sup>a</sup>The inter-assay precision was analyzed in triplicate in 3 separate run.

<sup>b</sup>The intra-assay precision was assessed by analyzing 3 replicates of each sample in a single run.

of  $0.98 \pm 0.13$  mg/mL indicated that the anti-complement potency of BCPs was comparable to that of BPs.

#### 4. Discussion

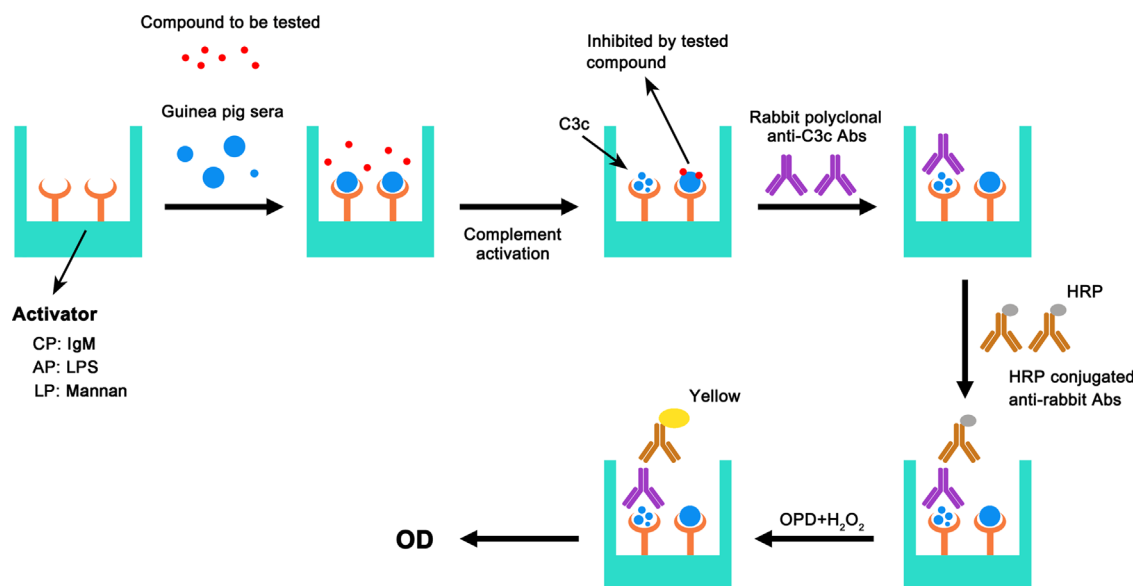
In our study, a C3c-based ELISA assay was developed and optimized for the determination of the inhibitory potency of complement inhibitors against the classical and lectin activation pathway. A schematic representation of the assay performance is shown in Fig. 3.

Although the hemolytic assay remains as the golden standard in the determination of inhibitory potency for complement inhibitors, it is not applicable for the study of the LP. Due to the emerging roles of the LP in clinical organ transplantation, infectious diseases, auto-immune disorders and link with the coagulation system<sup>20,21</sup>, it is of enormous importance to develop new assays to evaluate complement inhibitors against the LP. Our ELISA assay

fulfills this demand thus providing new insights into the development of anti-complementary compounds.

Moreover, this ELISA method is based on a 96-well plate, allowing a large number of compounds to be tested in a single run. Another advantage of our ELISA assay is that it is suitable for the screening of colorful compounds. For the hemolytic assay, the color of the compounds has an effect on the readout. Therefore, control groups are needed to eliminate the background color of the compound, which means larger amount of compounds are needed. The ELISA method described here, on the other hand, is not affected by the color of the compounds since they get washed off during the process. In that case, less amount of compounds is needed for the test, which is a big advantage especially when the compounds to be tested are only available in small quantity as they are difficult to be synthesized or extracted.

Compared to the hemolytic assay, our ELISA method is more reliable as the components used are commercially available and hence the quality is assured. Also it has better reproducibility and there is no batch-to-batch variation. In the hemolytic assay, sheep



**Figure 3** Schematic of the C3c-based ELISA assay for complement inhibitor screening. Firstly, 96 well plates are coated overnight with activation substrates for each respective pathway. After washing and blocking, pre-incubated mixture of guinea pig serum and the compound to be tested are added into the wells. Next, rabbit polyclonal anti-C3c Abs will detect complement activation by binding to bound C3c, followed by HRP-conjugated anti-rabbit Abs. Enzyme activity of HRP is assessed by measuring optical density (OD) after addition of OPD and H<sub>2</sub>O<sub>2</sub>. Inhibitory effect is reflected by decreased OD compared with control group (without tested compounds).

erythrocytes (SRBC) become increasingly fragile over time. For optimal viability, the blood cells should be used within 7–14 days. The source of SRBC varies, so it adds variation to the results from batch to batch. However, the guinea pig serum used in our assay can be prepared on a large scale and stored in  $-80^{\circ}\text{C}$  for a long period.

Additionally, with available antibodies, this assay can be adapted to capture other activation components in the complement system, such as the membrane attack complex instead of C3c. These adapted ELISA methods capturing different activated components might provide better insight into the mechanism of action of the inhibitors.

However, in our ELISA assay, the activation of complement *via* the AP is much lower than the other two pathways. Even at a serum concentration as high as 1:2.5, the OD values were less than 0.25 with a low signal-background ratio. For the lower concentrations of sera, there was no activation at all. In our study, we used LPS from *Escherichia coli* instead of *Salmonella typhosa* which was used by Roos et al.<sup>4</sup>. Researchers showed that LPS purified from different species, like *E. coli*, *S. typhosa* or *Pseudomonas aeruginosa*, have different stimulatory effects on cytokine and chemokine production due to their specific bacterial serotypes<sup>22</sup>. Therefore, they might also behave differently when used as an activator for the complement activation. In addition, they might also have different binding affinity to the 96-well plate. Future study will be carried out using other serotypes of LPS, trying to design and establish an appropriate ELISA method for the alternative pathway.

For the validation of the method, instead of the traditionally used complement inhibitor heparin, we used another well-studied inhibitor suramin. As a biological agent, heparin has a compromised stability and consistency. Our pilot experiments also showed that heparin did not behave consistently against the CP in ELISA method and no dose-dependent inhibition was observed (data not shown). Suramin on the other side is a synthesized

compound with good stability. It has been indicated to exhibit inhibitory effect in the complement system as it targets at various components including C1, C2, C4 and serine proteases<sup>19</sup>. For the CP, our ELISA assay showed an IC<sub>50</sub> of 0.089 mg/mL, which is in agreement with a literature report of a CH<sub>50</sub> around 0.1 mg/mL determined by hemolytic assay<sup>19</sup>. For the LP, Fong et al.<sup>19</sup> reported that suramin exhibited 90% inhibition on serine proteases at 1 mmol/L, suggesting it had some inhibitory effects on the LP considering the important role serine proteases play in the LP. However, so far no inhibitory effect on the full LP has been reported due to the lack of an adequate assay. Our paper for the first time reported that suramin indeed has inhibition on the LP with an IC<sub>50</sub> around 0.368 mg/mL. Validated by suramin, our ELISA assay showed a good precision and reproducibility.

In addition to synthesized compounds, we also used colored natural products *Bupleurum* polysaccharides to examine whether our assay is suitable for natural products, especially colored ones. Radix Bupleuri, known as ‘Chai-Hu’, is one of the most frequently prescribed crude herbs in traditional Chinese medicine for the treatment of inflammatory diseases. Our previous experiments demonstrated that the crude polysaccharides isolated from *B. smithii* and *B. chinense* showed inhibitory properties towards complement activation, involving both CP and AP<sup>8,23</sup>. However, their inhibitory potency on the LP was not studied. Therefore, we would like to determine the IC<sub>50</sub> on the LP for these two crude polysaccharides. Our ELISA assay showed that both BPs (IC<sub>50</sub> = 1.057 mg/mL) and BCPs (IC<sub>50</sub> = 0.098 mg/mL) have inhibitory effects on the LP and can be further studied for their application in LP related diseases such as rheumatoid arthritis and schizophrenia<sup>24,25</sup>. Together with the previous research results (BPs: CH<sub>50</sub> = 0.34 mg/mL, AP<sub>50</sub> = 0.081 mg/mL<sup>23</sup>; BCPs: CH<sub>50</sub> = 0.35 mg/mL, AP<sub>50</sub> = 0.337 mg/mL<sup>8</sup>), the inhibitory profiles of BPs and BCPs on the three complement activation pathways are complete. These data demonstrated that both BPs

and BCPs had strong anti-complementary properties on all three activation pathways. However, their targets against the CP and AP were different. BPs showed interaction with C1s, C3 and C4<sup>23</sup>, while BCPs blocked C1q, C2, C5 and C9<sup>8</sup>, suggesting that the mechanisms for their anti-complementary effects might be different and diverse. More comprehensive experiments, which allow direct assessment of the interaction between these two polysaccharides and their targets in the lectin activation cascade, are required to further demonstrate the similarity and difference between BPs and BCPs.

In conclusion, the ELISA assay described in this study is a simple high throughput screening approach for the determination of complement inhibitors. The assay is robust and reliable based on the validation results. Most importantly, it might be a promising approach to further study the mechanisms of complement inhibitors. We also showed that the previously identified CP and AP inhibitors suramin, *Bupleurum smithii* var. *Parvifolium* polysaccharides and *Bupleurum chinense* polysaccharides also have good anti-complement potency on the LP.

### Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (Nos. 81274165, 81330089 and 30925042), the State Key Program for Innovative Drugs from the Ministry of Science and Technology of the people's Republic of China (No. 2012ZX09301001-003), the Science and Technology Commission of Shanghai Municipality (Nos. 12JC1400800 and 10XD1405900), and Fudan's Undergraduate Research Opportunities Program (Xiyuan, No. 102305).

### References

1. Trouw LA, Daha MR. Role of complement in innate immunity and host defense. *Immunol Lett* 2011;**138**:35–7.
2. Amara U, Rittirsch D, Flierl M, Bruckner U, Klos A, Gebhard F, et al. Interaction between the coagulation and complement system. *Adv Exp Med Biol* 2008;**632**:71–9.
3. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. *Nat Immunol* 2010;**11**:785–97.
4. Roos A, Bouwman LH, Munoz J, Zuiverloon T, Faber-Krol MC, Fallaux-van den Houten FC, et al. Functional characterization of the lectin pathway of complement in human serum. *Mol Immunol* 2003;**39**:655–68.
5. Herpers BL, de Jong BA, Dekker B, Aerts PC, van Dijk H, Rijkers GT, et al. Hemolytic assay for the measurement of functional human mannose-binding lectin: a modification to avoid interference from classical pathway activation. *J Immunol Methods* 2009;**343**:61–3.
6. Cedzynski M, Swierzko AS, Kilpatrick DC. Factors of the lectin pathway of complement activation and their clinical associations in neonates. *J Biomed Biotechnol* 2012;**2012**:363246.
7. Wallis R. Interactions between mannose-binding lectin and MASPs during complement activation by the lectin pathway. *Immunobiology* 2007;**212**:289–99.
8. Di H, Zhang Y, Chen D. An anti-complementary polysaccharide from the roots of *Bupleurum chinense*. *Int J Biol Macromol* 2013;**58**:179–85.
9. Zwirner J, Wittig A, Kremmer E, Götze O. A novel ELISA for the evaluation of the classical pathway of complement. *J Immunol Methods* 1998;**211**:183–90.
10. Fredrikson GN, Truedsson L, Sjöholm AG. New procedure for the determination of complement deficiency by ELISA. Analysis of activation pathways and circumvention of rheumatoid factor influence. *J Immunol Methods* 1993;**166**:263–70.
11. Petersen SV, Thiel S, Jensen L, Steffensen R, Jensenius JC. An assay for the mannan-binding lectin pathway of complement activation. *J Immunol Methods* 2001;**257**:107–16.
12. Sarma JV, Ward PA. The complement system. *Cell Tissue Res* 2011;**343**:227–35.
13. Webster RO, Larsen GL, Henson PM. *In vivo* clearance and tissue distribution of C5a and C5a des arginine complement fragments in rabbits. *J Clin Invest* 1982;**70**:1177–83.
14. Teisner B, Brandslund I, Grunnet N, Hansen LK, Thellesen J, Svehag SE. Acute complement activation during an anaphylactoid reaction to blood transfusion and the disappearance rate of C3c and C3d from the circulation. *J Clin Lab Immunol* 1983;**12**:63–7.
15. Palarasah Y, Skjoldt K, Brandt J, Teisner B, Koch C, Vitved L, et al. Generation of a C3c specific monoclonal antibody and assessment of C3c as a putative inflammatory marker derived from complement factor C3. *J Immunol Methods* 2010;**362**:142–50.
16. Bier OG, Leyton G, Mayer MM, Heidelberger M. A comparison of human and guinea pig complements and their component fractions. *J Exp Med* 1945;**81**:449–68.
17. Trouw LA, Seelen MA, Duijs JM, Wagner S, Loos M, Bajema IM, et al. Activation of the lectin pathway in murine lupus nephritis. *Mol Immunol* 2005;**42**:731–40.
18. Jiang YW, Li H, Zhang YY, Li W, Jiang YF, Ou YY, et al. Beneficial effect of *Bupleurum* polysaccharides on autoimmune-prone MRL-lpr mice. *Clin Dev Immunol* 2012;**2012**:842928.
19. Fong JS, Good RA. Suramin—a potent reversible and competitive inhibitor of complement systems. *Clin Exp Immunol* 1972;**10**:127–38.
20. de Cordoba SR, Tortajada A, Harris CL, Morgan BP. Complement dysregulation and disease: from genes and proteins to diagnostics and drugs. *Immunobiology* 2012;**217**:1034–46.
21. Degn SE, Jensenius JC, Bjerre M. The lectin pathway and its implications in coagulation, infections and auto-immunity. *Curr Opin Organ Transplant* 2011;**16**:21–7.
22. Mathiak G, Kabir K, Grass G, Keller H, Steinringer E, Minor T, et al. Lipopolysaccharides from different bacterial sources elicit disparate cytokine responses in whole blood assays. *Int J Mol Med* 2003;**11**:41–4.
23. Xu H, Zhang Y, Zhang J, Chen D. Isolation and characterization of an anti-complementary polysaccharide D3-S1 from the roots of *Bupleurum smithii*. *Int Immunopharmacol* 2007;**7**:175–82.
24. Ammitzboll CG, Thiel S, Ellingsen T, Deleuran B, Jorgensen A, Jensenius JC, et al. Levels of lectin pathway proteins in plasma and synovial fluid of rheumatoid arthritis and osteoarthritis. *Rheumatol Int* 2012;**32**:1457–63.
25. Mayilyan KR, Arnold JN, Presanis JS, Soghoian AF, Sim RB. Increased complement classical and mannan-binding lectin pathway activities in schizophrenia. *Neurosci Lett* 2006;**404**:336–41.