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Development of an Immunoassay Platform for Diagnosis of Acute Kidney Injury

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***Abstract*—**Acute kidney injury (AKI) is a new worldwide public health problem. A diagnosis of this disease using creatinine is still a problem in clinical practice. Therefore, a measurement of biomarkers responsible for AKI has received much attention in the past couple years. Cytokine interleukin-18 (IL-18) was reported as one of the early biomarkers for AKI. The most commonly used method to detect this biomarker is an immunoassay. This study used a planar platform to perform an immunoassay using fluorescence for detection. In this study, anti-IL-18 antibody was immobilized onto a microscope slide using a covalent binding method. Make-up samples were diluted at the concentration between 10 to 1000 pg/ml to create a calibration curve. The precision of the system was determined using a coefficient of variability (CV), which was found to be less than 10%. The performance of this immunoassay system was compared with the measurement from ELISA.

***Keywords*—**Acute kidney injury, Acute renal failure, Antibody immobilization, Interleukin-18

I. Introduction

Acute kidney injury (AKI) or acute renal failure (ARF) is characterized by a reduction of renal function over time. AKI results in failure of the kidney to excrete nitrogenous waste products and to maintain fluid and electrolyte homeostasis [1]. It has been shown that renal ischemia injury is the leading cause of AKI [2]. The fatality rate of hospitalized patients with acute kidney injury has increased in the recent years, leading to a worldwide public health problem.

In current clinical practice, acute kidney injury (AKI) is normally diagnosed by the measurement of an increase in serum creatinine [3]. However, this method is not sufficiently sensitive and reliable because many factors such as age, sex, body mass and nutritional status which are not directly related to kidney damage may influence the changes in serum creatinine [4], [5]. In addition, the serum creatinine concentration does not increase until about half of the kidney function is lost [6], leading to the late diagnosis of AKI [6]. Hence, several biomarkers are being evaluated for early diagnosis of AKI.

Cytokine interleukin-18 (IL-18) has been reported as an early biomarker for diagnosis of AKI in the critical care setting [7]. It is a mediator of inflammation and ischemic tissue damage in many organs. IL-18 is formerly known as interferon-γ-inducing factor. It is a proinflammatory cytokine that is induced and cleaved as a 24 kDa inactive precursor in the proximal tubule [8]. Significantly high concentration of this biomarker was found in AKI pateints, while its concentrations in healthy controls or in patients with other renal disease were quite low [9].

The concentration of circulating biomarkers can be currently measured with conventional automated assays usually via enzyme-linked immunosorbent assay (ELISA, well-based platform) [10]. Although ELISA provides precise and accurate results, it requires large amount of reagent and is time-consuming [11], [12]. Therefore, an immunoassay in a planar platform has been introduced to overcome problems with high reagent consumption. In addition, the binding of antigen-antibody is believed to be more efficient in a microscale system, as a result of high surface area to volume ratio [13].

The most commonly used method to immobilize antibodies onto a planar platform is physical adsorption of proteins on a nitrocellulose membrane [12]. This method does not required any modification of antibodies and gives high-level antibody loading [14]. Nevertheless, antibody immobilization on nitrocellulose membrane results in large background noise, leading to high detection limit [15]. An alternative method for antibody immobilization is covalent binding of antibodies on a solid substrate. This method has been shown to be stable with strong attachment [16]. Several capture antibodies have been immobilized onto solid substrates for the following analytes, including T4, TSH , IL-1b, IL-2, IL-10, IL-6 MIP-1b, TGF-b, TNFa, VEGF and E-selectin [17], [18]. However, to the best of our knowledge, there is no planar immunoassay platform to measure IL-18 for early detection of AKI.

In this study, capture IL-18 antibody was immobilized onto a modified glass slide (planar platform) using a covalent binding method via 1 ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxy succinimide (NHS). The calibration curve was generated from makeup samples to determine the sensitivity of this system. The reliability and reproducibility of this assay system was investigated using inter- and intra assay techniques. The performance of our detection system was compared with that of ELISA.

II. Materials and Methods

A. Materials

Microscope slides were purchased from Sip Texnet I&E Medical (Sail brand, Jiangsu, China; 25.4 x 76.2 x 1 mm; Cat. 7101). Sodium hydroxide was purchased from Merck.

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Hydrogenperoxide (H_2O_2) was purchased from Carls Brba. Sulfuric acid (H_2SO_4) was purchased from J.T.Baker. Acetone and sodium hydrogen phosphate (Na_2HPO_4) was purchased from Ajax. 3-aminopropyltriethoxysilane (APTES), succinic acid anhydride (SAA), 1 ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and bovine serum albumin (BSA) were purchased from Sigma. N-hydroxy succinimide (NHS) was purchased from Fluka. Citric acid monohydrate was purchased from Riedel-de Haën. Anti-human IL-18 antibody, recombinant human IL-18, biotin labeled anti-human IL-18 antibody and northernlights^{TM}-labeled streptavidin were purchased from R & D Systems (Minneapolis, MN, USA).

B. Preparation of Glass Slides and Surface Modification

Glass slides were cleaned in 1M NaOH for 20 minutes, followed by a mixture of 30% H_2O_2 and 70% concentrated H_2SO_4 for 30 minutes. The slides were then carefully rinsed with water and dried under nitrogen flow. The cleaned glass slides were treated with 2% APTES in acetone for 2 minutes. After removal of the solution, the slides were rinsed several times with acetone and water to remove unbound silane and dried in an oven at 80 °C for 1 h. the dried slides were immersed in succinic acid anhydride pH 6.0 to produce carboxyl groups on the surface for 2 hours. Afterwards, the slides were rinsed with phosphatecitrate buffer and dried under nitrogen flow. The change in the surface chemistry was observed by the change in the surface's contact angle using a sessile drop method.

C. Antibody Immobilization on a Glass Slide

A 0.5 µl mixture solution containing 400 µg/ml of EDC, 600 µg/ml of NHS and 5 µg/ml of Anti-human IL-18 was spotted onto the modified slide surface using a manual pipette. The center-to-center spacing between spots was 5mm. Phosphatecitrate buffer pH 4.6 was used as a reaction buffer. The cross-linking process was allowed for 1 hour at room temperature, and then Tris-HCl buffer was used to terminate the reaction. After washing, the antibody immobilized slide was immersed with 1% BSA in PBS for 1 hour at 37°C to block any non-specific protein adsorption before being rinsed with PBS.

D. Immunoassay on a Glass Slide

The immunoassays were conducted following the procedure for sandwich-type assays. The recombinant human IL-18 sample was added to an antibody immobilized glass slide and incubated for 1 hour, followed by rinsing with PBS. Subsequently, anti-human IL-I8 biotinylated antibody diluted at 1:1000 was added and incubated for 1 hour. The slide was washed with PBS and the anti-human IL-I8 biotinylated antibody was hybridized with Northernlight^{TM} 493-strepavidin diluted at 1:3000 and incubated for 30 minutes at room temperature in the dark. In a negative control experiment, all steps were identical, except that the recombinant human IL-18 incubation step was omitted. The glass slide was imaged with Typhoon Trio scanner (Amersham Pharmacia Biotech, USA) using 488 nm excitation with 520 BP emission filters. The intensity of fluorescence was analyzed with ImageQuant^{TM} software (Amersham Pharmacia Biotech, USA). The fluorescence intensity of each spot was then calculated by subtracting the intensity obtained from the negative control.

E. Enzyme-Linked Immunosorbent Assay

A human IL-18 ELISA kit (R & D Systems, Minneapolis, MN) was used to detect recombinant human IL-18. Briefly, the samples were added (100 µl/well) to the antibody coated microwell and incubated for 1 hour. The plate was washed four times. Peroxidase conjugated anti-Human IL-18 antibody was subsequently added (100 µl/well) and incubated for 1 hour. The washing step was repeated. A substrate solution (100 µl/well) for peroxidase (3,3',5,5'-tetramethyl benzidine and H_2O_2) was added before incubating for 30 minutes at room temperature. The reaction was stopped by addition of 0.5 mol/L H_2SO_4 (100 µl/well). The plate was read using a wavelength of 450 nm in a microplate reader (Tecan Group, Switzerland).

III. Results and Discussion

A. The chemistry of the surface modification and antibody binding

The change of the glass's surface chemistry was observed using contact angle values as shown in Table I. The contact angle of the cleaned glass slide was 17.5°, indicating a hydrophilic surface. It shows that any contaminants on the surface were completely removed. After the treatment with APTES, the water contact angle of the surface increased to 56.7°, as a result of the deposition of silane layer on surface. Then, glass slide was treated with succinic acid anhydride to generate the carboxyl group. The contact angle decreased from 56.7° to 49.6°. The surface became more hydrophilic due to carboxyl group. The surface contact angle increased to 75° after the immobilization of anti-human IL-18 antibody via the reaction between carboxyl group from slide's surface and the amino group of the antibody. This result shows that antibody was successfully immobilized onto the glass slide.

TABLE I
Water Contact Angle of Cleaned, Modified and Immobilized Glass Slide

Sample	Cleaned	APTES-modified	SAA-modified	Immobilized
Contact angle	17.5°	56.7°	49.6°	75°

B. Optimized Capture Antibody Concentration

The antibody specific to recombinant human IL-18 was immobilized on a surface-treated glass slide at various concentrations of 2, 5 and 10 µg/ml to determine the optimized concentration of the antibody for an immunoassay. The immobilization mechanism is based on covalent bonding between the amino groups of the antibody and carboxyl-terminated surface of the modified glass slide. As shown in Fig. 1, the maximum fluorescence intensity was observed when 5 µg/ml of anti-IL-18 antibody was used for the antibody immobilization. The fluorescence intensity at 10 µg/ml was lower than that of 5 µg/ml because the steric hindrance near the immobilized antibody affected the antigen

binding sites leading to a decreased antibody activity [19]. Therefore, 5 µg/mL of capture antibody was used for further studies.

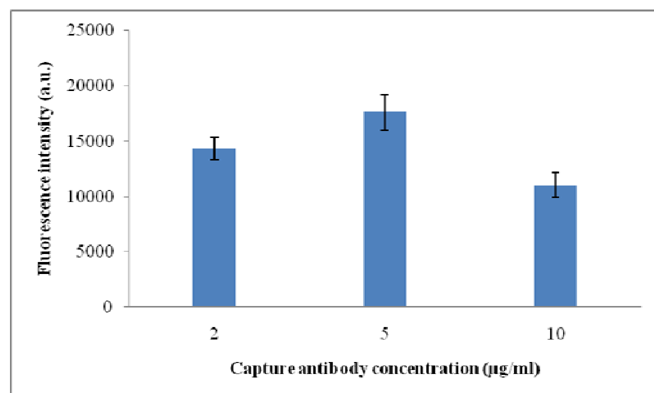

Fig.1 Fluorescence intensities when the concentrations of immobilized antibody specific for IL-18 were 2, 5, and 10 µg/mL

C. The Design of a Glass Slide Platform and the Calibration Curve of IL-18

Samples containing recombinant human IL-18 were diluted in PBS at a range of concentrations between 10 to 1,000 pg/ml. The measurement of each concentration was carried out in 6 replicates. The immunoassay experiment was designed as shown in Table II. "Blank" indicates spots where there was no capture antibody immobilized to determine the noise level, while "neg." represents the spots where samples containing no antigen were incubated with immobilized antibody to determine the background signal. Figure 2a shows a schematic diagram of our antibody-immobilized glass slide. The diameter of each spot was approximately 1.2 ± 0.1 mm with 5 mm spacing. There are 90 spots within one slide, allowing for 90 experiments including blank, negative and positive controls. A representative fluorescent image of the immunoassay glass slide is shown in Figure 2b. The fluorescent labeled antibodies were clearly binding to the targets on the glass slide. Nonspecific binding outside the immobilized antibody spots were not observed. The background signal calculated from the negative control spots were below a detectable level. As expected, higher concentrations of the analyte resulted in higher fluorescent intensity.

TABLE II
POSITION ON MICROSCOPE SLIDE

	A	B	C	D	E	F
1	blank	blank	blank	blank	blank	blank
2	blank	neg.	10	25	50	blank
3	blank	neg.	10	25	50	blank
4	blank	neg.	10	25	50	blank
5	blank	neg.	10	25	50	blank
6	blank	neg.	10	25	50	blank
7	blank	neg.	10	25	50	blank
8	blank	blank	blank	blank	blank	blank
9	blank	100	200	500	1000	blank
10	blank	100	200	500	1000	blank
11	blank	100	200	500	1000	blank
12	blank	100	200	500	1000	blank
13	blank	100	200	500	1000	blank
14	blank	100	200	500	1000	blank
15	blank	blank	blank	blank	blank	blank

neg. = negative control, pos. = positive control
The unit of concentration is pg/ml.

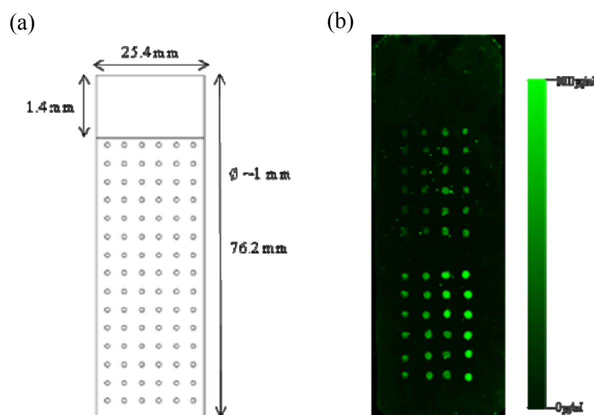

Fig. 2 (a) Schematic design of slide, (b) Fluorescene image

The calibration curve of IL-18 measured from the immunoassay glass slide is plotted as shown in Figure 3. The fluorescent intensity is linearly proportional to the recombinant human IL-18 concentration between 10 pg/ml to 1000 pg/ml in the test sample. The correlation coefficient (R^2) of this model is 0.989, which is considered to be quite high. The sensitivity or the lower detection limit (LOD) of this immunoassay calculated based on the signal to noise ratio of 3 is 15 pg/ml. The concentration of IL-18 in normal individuals and patients with AKI is >10 pg/ml and >100 pg/ml, respectively [20-23], which is within the range that this method can detect. Therefore, this method has potential to quantify the concentration of IL-18 for early diagnosis of AKI.

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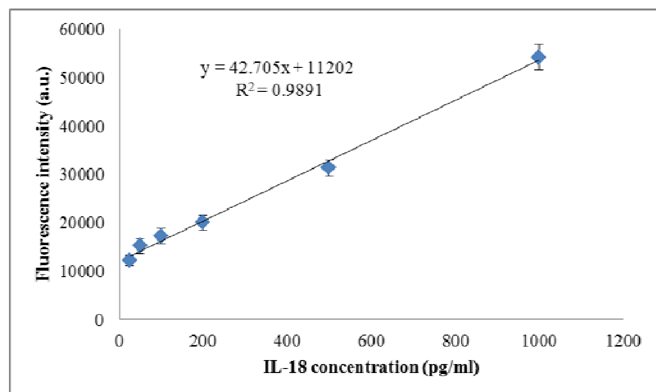

Fig. 3 Calibration curve of IL-18

D. Precision Assay

The precision, or repeatability, of the immunoassay test results can be expressed using two measures of the coefficient of variability (CV): the inter-assay CV and the intra-assay CV. The CV is a dimensionless number defined as shown in equation (1) [24]. The intra-assay is used to determine the spot-to-spot variability, while the inter-assay shows the slide-to-slide reproducibility.

$$\%CV = \frac{\text{Standard deviation}}{\text{Mean}} \times 100 \quad (1)$$

Three samples of known concentrations (50,100, and 200 pg/ml) were tested twenty times on one slide to assess the intra-assay precision. Twenty replicates of each sample in five separate assays were used to assess the inter-assay precision. The intra-assay CV among the 6 spots of each concentration was, ranged from 2.45% for 200 pg/ml to 7.77% for 50 pg/ml and the average of the intra-assay CV was 6.42%. The error of the measured concentrations from the actual concentrations calculated using the equation (2) was ranged from 0.63% to 8.46% and the average of the error was 3.53%. The summary of the results is shown as Table III.

$$\%Error = \frac{|\text{measured value} - \text{actual value}|}{\text{actual value}} \times 100 \quad (2)$$

TABLE III
INTRA-ASSAY

Sample	1	2	3
Actual concentration (pg/ml)	50	100	200
n	20	20	20
Mean (pg/ml)	54.23	98.50	198.74
%CV	7.77	9.03	2.45
%Error	8.46	1.50	0.63

CV = coefficient of variability

For the inter-assay, The CVs were 1.32% for 200 pg/ml, 2.81% for 50 pg/ml, and 4.96% for 100 pg/ml. The average of the inter-assay CVs was 3.03%. The error of the measured concentrations from the actual concentrations was ranged from 1.02% to 6.96% and the average was 4.20 %. The data is shown in Table IV.

TABLE IV
INTER-ASSAY

Sample	1	2	3
Actual concentration (pg/ml)	50	100	200
n	20	20	20
Mean (pg/ml)	53.48	104.63	202.03
%CV	2.81	4.96	1.32
%Error	6.96	4.63	1.02

CV = coefficient of variability
From twenty (20) replicates of each serum sample in five (5) separate assays

According the research of biological studies [25] and the manufacture's instruction (Salimetrics, USA), the intra-assay CV of less than 10% is generally acceptable. The inter-assay CV should be less than 15%. The intra- assay of this method is below 10% and the inter-assay is well-below 15%. Thus, the CVs of the intra-, and the inter-assay of this technique indicate reliable detection. Moreover, the % error of this measurement is less than 10, which is considered acceptable. As a result, this immunoassay system can be used to accurately analyze the recombinant human IL-18.

E. Comparison between ELISA and Glass Slide System for IL-18 analysis

Various concentrations of recombinant human IL-18 were analyzed with an ELISA kit and compared with those measured from the glass slide system. The calibration curve generated from immunoassays using ELISA was compared with that of the glass slide system at the same concentrations (Table V). The concentrations of the recombinant human IL-18 measured from the glass slide platform were quite close to the actual concentrations. At some concentrations, the measurement from our system is better than that of ELISA. According to Table VI, the R^2 of the ELISA method (0.9982) is better than that of the glass slide system (0.9891). The sensitivity of ELISA is 12.5 pg/ml as shown in the manufacture's instruction whereas that of glass slide is 15 pg/ml. The slight difference in the limit of detection indicates that this glass-slide system is quite sensitive. The efficiency of the planar platform is approximately 80% when compared with the conventional method. Although the efficiency of this platform is not equal the conventional method, the glass slide system has demonstrated its possibility to be used to perform an immunoassay for IL-18 analysis. Furthermore, the reagent consumption in this system is 200 times less than that of the traditional assay in a well-based format.

TABLE V
COMPARISON BETWEEN THE ACTUAL, ELISA AND GLASS SLIDE VALUE

Actual concentration (pg/ml)	ELISA (pg/ml)	Glass slide (pg/ml)
25	15.58	22.72
50	64.75	75.26
100	83.92	124.98
200	183.50	205.77
500	516.42	470.51
1000	974.25	1007.39

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TABLE VI
COMPARISON BETWEEN ELISA AND GLASS SLIDE

	ELISA	Glass slide
Volume of reagents	100 µl/sample	0.5 µl/sample
Sensitivity	12.5 pg/ml	15 pg/ml
Correlation coefficient (R^2)	0.9982	0.9891

IV. CONCLUSIONS

The mortality and morbidity associated with AKI has become a clinical problem worldwide. A conventional diagnosis, such as the measurement of serum creatinine, is not sufficiently sensitive and early enough. Thus, several biomarkers have played an important role in early diagnosis of AKI. Cytokine interleukin 18 (IL-18), proinflammatory cytokine, has been reported as one of the biomarkers for early detection of AKI.

In this study, capture IL-18 antibody was immobilized onto a glass slide using EDC and NHS as linkers. The immunoassays performed on this platform gave satisfactory results. The calibration curve generated from this system shows a linear relationship in the range of 10 – 1000 pg/ml of IL-18 concentrations. Although the R^2 of this system was somewhat lower than that of ELISA assays, the limit of detection of the two systems were quite comparable. The coefficients of variations were within an acceptable limit, indicating good reproducibility and repeatability of this assay platform.

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