Screening Platform Based on Robolid Microplate for Immobilized Enzyme-Based Assays

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ABSTRACT: A facile, cost-effective, and high-throughput screening method was developed for enzyme-based assays based on Robolid/Microplate (RLMP) platform. The RLMP platform is constructed by immobilizing enzyme on commercial robolids and combining it with a standard 96-well microplate to achieve high-throughput analysis. The initiation and quenching of enzymatic reaction can be performed by simply sandwiching or unsealing the enzyme-immobilized robolids and the sample-containing microplate. This platform enables measurements of multiple target analytes simultaneously based on immobilized enzymatic reactions, with analysis time independent of the number of wells in the microplate. Using urea as the model analyte, we have shown that the RLMP platform exhibits large linear detection range of up to 10 mM, fast analysis time of 30 min/96 samples, as well as good reproducibility and stability. Measurements of urea in human urine and serum samples were performed using the RLMP platform and were compared with the commercial urea test kit. A good correlation was found between the two methods. This study shows that the present RLMP platform has promising prospects for detection of clinical markers and application in disease diagnosis and biochemical analysis.

1. INTRODUCTION

The need for simple, rapid, cost-effective, and high-throughput screening approaches has boosted the development of applications in practical clinical assays and disease diagnosis. Compared with traditional analytical methods, enzyme-based assays have several distinct advantages, such as high sensitivity and specificity, cost-effectiveness, and the possibilities for miniaturization and mass production. Thus, enzyme-based assays are of great value for qualitative and quantitative analysis of a variety of target analytes in biomedicine and clinical diagnosis.1−5 It has been shown that the elevated enzyme activities in clinical samples provide important information about the disease and treatment options6−8 and that enzyme-catalyzed reactions provide efficient assays for a variety of biomarkers for clinical diagnosis covering nitrogenous metabolites,9−10 carbohydrates and carbohydrate metabolites,11−14 lipids and lipoproteins,11,12 and so forth.

Compared to free enzyme, the applications of enzyme immobilization can improve the stability and reusability of an enzyme as well as greatly reduce experimental cost, sample manipulation, and analysis time.15 Thus, enzyme immobilization has played an important role in developing enzyme-based biosensors.14,15 A number of techniques have been developed for biosensor applications with immobilizing enzymes as well as cells on different support matrices, such as functionalized polymers, biocompatible composite materials, nanomaterials, and sol−gel and hydrogel materials.16,17 Despite these significant advantages, however, it remains a quite challenging task for immobilized enzyme-based biosensors to accomplish high-throughput assays for multiple samples, which is essential to meet the growing demand for clinical diagnostics. One approach to overcome this issue is by integrating the microchip devices with microarray screening technologies. Such microarray approach can offer the ability to perform assays on array platforms consisting of thousands of reaction spots, thereby greatly increasing throughput.18−20 However, the sophisticated microarray fabrication, in a manner, impedes analysis of these arrays, particularly for real-time analysis and application popularization.

In the present study, we aim to develop a facile, cost-effective method for high-throughput screening assays based on immobilized enzyme biosensors. Commercially available robolids of a 96-well microplate are employed as support for immobilization of enzymes. Robolid is a microplate seal that incorporates the silicone capmats into a standard polystyrene plate lid. We will show that the enzyme-modified robolids combined with a standard 96-well plate can be used as a robust high-throughput analysis platform, which is dubbed as the Robolid/Microplate (RLMP) platform in the present study. Figure 1A shows schematically the idea of the RLMP for high-throughput assays. We selected alginate−chitosan as a matrix for enzyme encapsulation and immobilized enzyme on the robolids to form the enzyme microreactors. Such robolid-based enzyme reactors then attach to each individual well of the 96-well plate to perform the assays for multiple samples (Figure 1A−a). Initiating (or quenching) the enzyme reactions in the
RLMP platform can be easily achieved by simply sandwiching (or turning over the plate) the enzyme-immobilized robolids onto the sample-containing wells (Figure 1 A-b,c). Adding quenching reagents or heating procedures is not necessary. Thus, complicated manipulation for controlling enzymatic reaction in traditional enzyme assays using a 96-well microplate can be avoided. After the enzymatic reactions are stopped, the microplate containing the reaction solution in each well is ready for various detection analyses (Figure 1A-d,e). The analysis is able to detect as much as 96 samples in a single plate simultaneously and makes the measurement time independent with the number of wells in the microplate. The robolids are commercially available, and enzyme-immobilized robolids can be reusable and cost-effective, which are particularly beneficial for clinical practice and promotion. We use urea as the model target analyte to evaluate the feasibility and performance of the proposed RLMP platform. As urea is the main end product of human protein metabolism, its

Figure 1. (A) Schematic diagram of RLMP assay process. (B) Fabrication procedure of the urease-RLMP platform for high-throughput urea determination.

Figure 2. Optimization of urease immobilization conditions for RLMP platform. Effects of sodium alginate concentration (A), curing time of sodium alginate (B), chitosan concentrations (C), and curing time of chitosan (D). Urease concentration was kept at 7 mg/mL. Urea solution (4.0 mM) was used as the test sample. Each data point in the figures was an averaged result of three repeatable assays. The immobilization yield represents the ratio of the specific activity of the immobilized enzyme to that of the free enzyme (in sodium alginate).
determination has become one of the most useful and direct ways of clinical diagnostics and medical care in the appraisal of renal functions. For urea measurements, the applied chemistry has been dominated by urease-catalyzed hydrolysis involving colorimetric or electrochemical detection.\(^{21–24}\) Urease catalyzes the hydrolysis of urea to carbamic acid and ammonia, which then decompose, respectively, to bicarbonate and another molecule of ammonia

\[
(\text{NH}_2)_2\text{CO} + 2\text{H}_2\text{O} + \text{H}^+ \rightarrow 2\text{NH}_4^+ + \text{HCO}_3^-
\]

In this work, measurements of urea in human urine and serum samples were performed by the RLMP platform, and the results were compared with commercial urea test kit.

2. RESULTS AND DISCUSSION

2.1. Optimization of the Urease-RLMP Platform. We first investigated and optimized several key experimental parameters involved in the fabrication process of the urease-RLMP platform, which may affect the enzyme reactivity. The results are presented in Figure 2. The immobilization yield in Figure 2 represents the ratio of the specific activity of the immobilized enzyme to that of the free enzyme (in sodium alginate). It is known that different sodium alginate concentrations can form gels with different strengths in the presence of \(\text{BaCl}_2\) and that the encapsulated enzyme activity would be affected by the gel strength. As shown in Figure 2A, the maximum immobilization yield was observed at 2.0% sodium alginate concentration. At this concentration, we found that the alginate/urease sol can easily form a hemispherical and uniform gel (diameter, 3 mm; height, 2 mm) with high strength. At lower concentration, however, the alginate gel was soft and flaccid and the urease leaching was increased according to the storage studies. On the other hand, with excessive increase of the concentration of sodium alginate, the diffusional resistances to the substrate offered by alginate gel were increased and resulted in a decrease of hydrolysis of urea. For the subsequent experiments, the concentration of sodium alginate was fixed at 2.0%. The gelation time of alginate with \(\text{BaCl}_2\) is another parameter for the assays using the urease-RLMP platform. It was found that, with increasing incubation time of alginate/\(\text{BaCl}_2\) from 0.5 to 2 h, the immobilization yield increases and then reaches its maximum value at 2 h. After that, the immobilization yield was reduced with further increase in the gelation time (Figure 2B), which could be attributed to overhigh diffusional resistances with large degree of gelation. The result is consistent with the report wherein alginate beads were formed and stirred for 2–3 h to generate rigidity when soybean urease was immobilized on alginate.\(^{25}\)

In Figure 2C, the effect of chitosan concentration on the immobilization yield is shown. Chitosan is a cationic polysaccharide obtained from partial chitin deacetylation. It can form polyelectrolyte complexes (PECs) with alginate. As shown in Figure 2C, the optimal concentration of chitosan for the assay was determined to be 2.0% in the present study. It is noted that, as the layer of chitosan on the surface of the alginate gel could cause the transfer resistance of the substrate to penetrate into the alginate/enzyme gel, it can greatly reduce the leakage of the enzyme and enhance the stability of the robolid enzyme reactor. Indeed, one can see from Figure 2D that there are no apparent differences for nine cycle-runs with the alginate/chitosan incubation time of 30 or 45 min. The stability and reusability of the urease microreactor will be further demonstrated in Figure 5 and will be discussed in the following section.

The amount of enzyme and the enzymatic reaction time for urea assay were further investigated. The urea concentration varied from 1.0 to 11.0 mg/mL. The activity of urease reached the maximum at 7 mg/mL and became almost constant at higher concentrations, as shown in Figure 3A. The effect of enzymatic reaction time on the absorbance of product of immobilized urease was studied by incubating the enzyme–substrate reaction mixture for 6–30 min. As shown in Figure 3B, with increasing incubation time, the absorbance of the product increases quickly at the initial step and then reaches its maximum value at 25 min. Considering both analysis speed and urea detection sensitivity, 10 min was set as the reaction time.

2.2. Performance of the Urease-RLMP Platform. Under these optimized conditions, the sensitivity and linear range of the proposed RLMP platform for urea assay were investigated.

Standard solutions with various concentrations of urea were used as substrate. The dose–response curve exhibits excellent linear dependence in a wide urea concentration range of 0.01–10 mM (Figure 4). The linear fitting result is \(A_{560} = 0.1541C + 0.0491\) (\(R^2 = 0.9958\)), where \(A_{560}\) and \(C\) represent the
absorption at 560 nm and the concentration of urea, respectively. The limit of detection (LOD) was determined by obtaining signals of multiple reagent blanks (n = 8) according to the equation:

$$\text{LOD} = \frac{3S}{A}$$

where S is the standard deviation of the blank measurements and A is the slope of the linear calibration curve obtained using standards ranging from 0.01 to 10 mM urea. The LOD was then calculated as 0.005 mM urea. Compared with other methods for urea measurement, in addition to its low LOD and large linear detection range, the RLMP platform exhibits fast analysis time, that is, up to 30 min/96 samples. The Michaelis constant ($K_m$) of immobilized urease was derived by the Lineweaver−Burk plot, as shown in Figure 4B. The $K_m$ value is determined to be 6.22 ± 0.23 mM using the RLMP platform, which is close to that by free urease (4.05 ± 0.31 mM). This result indicates that there is no significant structural change of the enzyme or reduction of accessibility of the substrate to the active sites of the immobilized urease in our fabricated RLMP platform.

Compared to free enzyme, one advantage for the application of enzyme immobilization is the reusability of the enzyme. In Figure 5A, we show that, using alginate−chitosan for immobilization, the enzyme can still maintain over 80% of its reactivity after nine continuous runs. The loss of activity of entrapped enzyme could be a result of the leakage of enzyme from the barium alginate−chitosan gels as results of washing of gels at the end of each cycle or conformational changes by repeated uses. If only alginate is used for enzyme immobilization, however, the reactivity drops to 57% after just five runs. The results indicate that the reusability of the enzyme reactor can be enhanced by using alginate−chitosan for immobilization in the RLMP platform. To demonstrate the reusability of the reactor for different microplates, we split the same human urine sample diluted 200-fold into eight microplates, followed by the successive tests using the same urease-immobilized robolids. Phosphate buffer was used as blank and added in the last two wells for each microplate. The results showed that the immobilized enzyme can still maintain over 78% of its reactivity after eight successive tests for different microplates.

The storage stability of free and immobilized urease is presented in Figure 5B. The free and immobilized enzymes were stored at 4 °C, and their activities were measured in 30 days. As shown in the figure, after 7 days, the robolid-based reactor retained 98% of its enzyme activity, whereas for free urease, the activity decreased to 88%. After the 30 day storage, the robolid-based reactor still presented 88% of its activity. This observation indicates that the immobilized urease on robolids exhibits greater stability than the free enzyme. These results could be explained by the fact that immobilization can reduce the interaction between enzyme molecules, leading to the deactivation of the enzyme activity.

Anti-interference properties are important considerations for the proposed method. The possible interferences of various biological and organic species in the urea determination were investigated, and the obtained results are shown in Figure 6. It was found that no significant interfering effects were found for urea solutions with interferents, after adding a sample of 1.0 mM urea solution with different interferents. Thus, the presence of these species did not influence urea determination.

2.3. Real Sample Analysis Using the RLMP Platform. To evaluate the reproducibility of the immobilized urease reactor for different vessels of 96-well microplate, standard urea solution of 1 mM concentration was added in 30 wells of the microplate, whereas a human urine sample diluted 200-fold and a human serum sample diluted 3-fold were added in the rest of wells (32 wells for each). Phosphate buffer was used as blank and added in the remaining two wells of the microplate. The

![Figure 4](image1.png)

**Figure 4.** Dose−response curve of urea (A) and Michaelis−Menten curve for urease-RLMP platform (B). Urease concentration was kept at 7 mg/mL.

![Figure 5](image2.png)

**Figure 5.** (A) Reusability of the robolid-based enzyme reactor using alginate−chitosan (red) or alginate (black) for immobilization. (B) Storage stability of the free and immobilized urease. Each data point in the figures is an averaged result of three repeatable assays.
results show good reproducibility in terms of product absorbance with relative standard deviation (RSD) values of 6.5% \( (n = 30) \), 6.7% \( (n = 32) \), and 6.8% \( (n = 32) \) for 1 mM standard urea, human urine, and serum samples, respectively.

To demonstrate the feasibility of the proposed RLMP platform for analytical application of urea detection, the recovery test was performed by the standard addition method. As shown in Table 1, the obtained recoveries ranged from 96.4 to 103.0% in the spiked ultrapure water, from 95.3 to 104.0% in the spiked human urine samples, and from 97.1 to 107.0% in the spiked human serum samples, with three different additions of urea to the ultrapure water and diluted urine and serum samples (urine diluted by 200-fold; serum diluted by 3-fold).

| sample                  | urea added (mM) | urea found (mM) | recovery (%) | RSD (%, \( n = 3 \)) |
|-------------------------|-----------------|-----------------|--------------|-----------------------|
| human serum (×3)        |                 |                 |              |                       |
| 0                       | 2.12            | 1.86            |              |                       |
| 1.0                     | 3.19            | 107.0           | 2.05         |                       |
| 4.5                     | 6.49            | 97.1            | 2.32         |                       |
| 7.6                     | 9.81            | 101.2           | 2.15         |                       |
| human urine (×200)      |                 |                 |              |                       |
| 0                       | 1.09            | 2.10            |              |                       |
| 1.0                     | 2.13            | 104.0           | 2.23         |                       |
| 4.5                     | 5.48            | 97.6            | 1.95         |                       |
| 7.6                     | 8.33            | 95.3            | 2.01         |                       |
| ultrapure water         |                 |                 |              |                       |
| 0                       | 0.08            | 1.54            |              |                       |
| 1.0                     | 1.11            | 103.0           | 1.48         |                       |
| 4.5                     | 4.42            | 96.4            | 1.65         |                       |
| 7.6                     | 7.87            | 102.0           | 1.83         |                       |

Measurements of urea in human urine and serum samples were performed by the RLMP platform, and the results were compared with urea assay kit using free urease. A total of 28 human urine samples from healthy volunteers were diluted 100, 150, and 200 times, whereas 28 human serum samples collected from Beijing Friendship Hospital were diluted 3, 6, and 9 times before analysis. The results are shown in Figure 7. The results of nearly all of the volunteers were in the normal urea range (155–380 mM$^{23}$ in human urine). For urea in serum samples, there are 8 samples out of the 28 volunteers that are beyond the normal range of 2.5–7.5 mM$^{-1}$ whereas the others are in the range. The insets in Figure 7 show the correlation between the results of our RLMP method and those of the clinical urea assay kit. The results show very good correlations for either the urine or serum samples, indicating that our RLMP platform is a reliable approach for urea determination in real samples.

3. CONCLUSIONS

A novel RLMP platform for simultaneous analysis of multiple samples has been reported, using commercially available robolids as support for immobilizing enzyme and a standard 96-well microplate for detection. The platform integrates the advantages of both enzyme immobilization and microplate analysis and thus exhibits several salient features for enzyme-based clinical assays, for example, high throughput, parallelization, and cost-effectiveness. The feasibility and performance of the proposed RLMP platform for clinical application have been evaluated using urea as the model target analyte. The results show that the platform is a reliable approach for urea determination in real urine samples with low LOD, large linear detection range, fast analysis time, good stability, and reproducibility. To further reduce the leakage of enzyme, a chemical process (e.g., cross-linking with glutaraldehyde) might be necessary to reduce the release of the enzymes from the hydrogel mesh. The present method can be easily extended to...
various kinds of enzyme-based assays as well as inhibition assays, by immobilizing different enzymes on the robolids. We should mention that the throughput of our RLMP platform is determined by the number of wells in a microplate. It is important to note that hundred- and even thousand-well microplates are now commercially available for clinical assays. Our study indicates that the RLMP platform has promising prospects for the detection of clinical markers and application in disease diagnosis and biochemical analysis.

4. EXPERIMENTAL METHODS

4.1. Instrumentation. The solution dispensation was performed using a 96-channel pipetting system (Medusa 96; D.C.Labware Ltd., Guangdong, China). Microplate analysis was performed by Epoch 2 Microplate Spectrophotometer (BioTek, Winooski, VT).

4.2. Materials and Reagents. Urease (E.C.3.5.1.5, Canavalia ensiformis), 96-well robolid and polystyrene microplate, chitosan (medium molecular weight; deacetylation degree, 75−85%) and poly-L-lysine (PLL; molecular weight, 150 000) were purchased from Sigma Chemical (St. Louis, MO). Urea was obtained from Aladdin (Shanghai, China). Urea Assay Berthelot kit, buffered chromogen (3% (w/v) phenol + 0.015% (w/v) sodium nitroprusside), and alkaline hypochlorite solution (1.5% (w/v) sodium hydroxide + 0.12% (w/v) sodium hypochlorite) were purchased from Beijing Leagene Biotechnology Co., Ltd. (Beijing, China). Sodium alginate (medium viscosity; molecular weight, 80−120 kDa; mannuronate/guluronate ratio, 0.6) was obtained from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). All other reagents were of analytical grade and used without further purification. Ultrapure water was used to prepare all solutions.

The human urine samples were collected from healthy volunteers and centrifuged for 20 min at a speed of 1000 rpm to remove bacteria and protein aggregates. The supernatants were filtered and diluted 100, 150, and 200 times with phosphate-buffered saline (PBS) before analysis. The human serum samples were collected from Beijing Friendship Hospital (Beijing, China) in accordance with the rules of the local ethical committee. The serum samples were diluted with PBS buffer three, six, and nine times before analysis.

4.3. Fabrication Process of the Urease-RLMP Platform. The commercial robolids of 96-well microplate made of polystyrene contain 96 silicone-made micropillars (pillar diameter, 5 mm; pillar-to-pillar distance, 2 mm). The fabrication process of the urease-RLMP platform is schematically shown in Figure 1B. The enzyme urease in alginate can be strongly attached on the silicone-made micropillars of the robolids through poly-L-lysine (PLL) and BaCl₂. PLL with amine groups was absorbed on the silicone surface and then negatively charged alginate (−COO⁻ groups) was gelled by Ba²⁺ and attached to positively charged PLL (−NH₃⁺ groups) by an ionic interaction. Chitosan as a polycationic polymer was used to control the disintegration of alginate gel, on the basis of the ionic interactions between −COO⁻ groups of alginate and −NH₃⁺ groups of chitosan. The operation involving reagent transfer and dispensation was performed with a 96-channel
pipetting system. The amount of NH₃ liberated in the wells was determined on the basis of the Berthelot reaction.³³,³⁴

A 96-well microplate, each well containing 50 μL of PLL at a concentration of 0.01% w/v, was first tightly sealed by robolids. The microplate was then turned over, allowing the micropillar surfaces of the robolids to be incubated in PLL for 0.5 h at room temperature to ensure the deposition of PLL on the micro-polymer pillar surface. After the microplate was detached, the robolids were washed with phosphate buffer and dried at room temperature. BaCl₂ (30 μL, 0.2 M) was simultaneously dispensed on the surface of each of the 96 PLL-coated micropillars using a 96-channel pipetting system. The robolids were then dried overnight at room temperature. To encapsulate urease in alginate on the robolids, 10 μL of urease–alginate mixture was spotted on the PLL/BaCl₂-coated robolids. The suspension of urease in low-viscosity alginate was prepared by mixing 7 mg of urease powder in 1 mL of 2.0% alginate solution and stirred for 1 h to ensure complete mixing. To increase the gelling of alginate and BaCl₂, the alginate-coated robolids were placed on a chilling deck at 12 °C for 2 h, the robolids were removed from the microplate and washed with phosphate buffer twice. Chitosan (30 μL, 2% (v/v)) was then dispensed on the tip of urease/alginate-coated micropillars, and the robolids were incubated on a chilling deck at 12 °C for 0.5 h. The application of chitosan can decrease the disintegration of the urease–alginate gel, based on the ionic interactions between the −COO⁻ groups of alginate and the −NH₃⁺ groups of chitosan, which can form polyelectrolyte complexes (PECs) between alginate and chitosan. After the urease immobilized, the robolids were washed with phosphate buffer and stored at 4 °C before use.

4.4. Urea Determination. For urea determination using the urease-RLMP platform, 80 μL of standard urea solutions with desired concentrations and diluted human urine or serum samples were added in the wells of a 96-well microplate, which was then sealed with the urease-immobilized robolids. The enzymatic reaction was started by turning over the microplate to mix the solution in each well with the immobilized urease on the surface of the robolids. After the enzyme reactions were performed for 10 min at 37 °C, the RLMP platform was turned over again and the robolids were separated from the microplate to stop the enzymatic reactions. For measuring the amount of ammonium produced, 80 μL of buffered chromogen and 80 μL of alkaline hypochlorite for Berthelot reaction were added to each well of the microplate. After 20 min derivatization reaction at 37 °C, absorbance at 560 nm was recorded using Epoch 2 Microplate Spectrophotometer.

The urea determination in human urine and serum samples was also performed using commercial urea assay Berthelot kit. Briefly, 40 μL of standard solutions of urea with desired concentrations and diluted human urine or serum samples were added into the wells of 96-well microplate, followed by adding 40 μL of urea working solution, which was dissolved in 50 mM phosphate buffer (pH 7.0). After the reaction was carried out at 37 °C for 10 min, the amount of ammonium was measured by adding 80 μL of buffered chromogen and 80 μL of alkaline hypochlorite to each well and absorbance at 560 nm of reaction solution was recorded used Epoch 2 Microplate Spectrophotometer after 20 min incubation for derivatization reaction.

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