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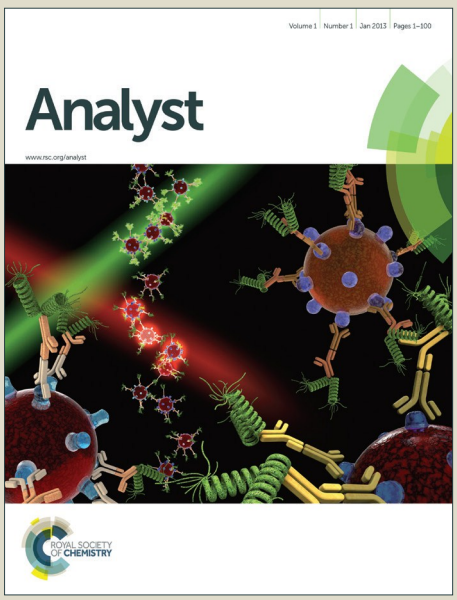

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A Microfluidic Indirect Competitive Immunoassay for Multiple and Sensitive Detection of Testosterone in Serum and Urine

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We demonstrate a microfluidics-based indirect competitive chemiluminescence enzyme immunoassay (MIC) for multiple, sensitive, reliable and rapid detection of testosterone in human serum and urine samples. As MIC can detect biomarkers in a cost-effective and easy-to-operate manner, it may have great potential for clinical diagnosis and point-of-care testing (POCT).

Introduction

Testosterone is a steroid hormone, which is secreted by the testicles of males, the ovaries of females, and the adrenal glands. The level of testosterone is related to the growth of muscle mass and strength, the stimulation of bone growth, and the maturation of sex organs.[1, 2] Recent studies consider low testosterone level as a possible risk factor for dementia of the Alzheimer type.[3] A high testosterone level may be associated with menstrual cycle irregularities in women,[4] while the slightly high testosterone level in men may reduce the risk of high blood pressure or heart attack.[5] Since the above effects are mostly dependent on the level of circulating free testosterone, the accurate and rapid detection of testosterone in peripheral blood is of great significance. In addition, the abuse of testosterone could improve muscle strength and athletic performance, which is strictly prohibited by most sports regulators.[6] To date most methods for detecting drug abuse in athletes are based on urine tests, so that a direct and rapid assay for testosterone detection in urine samples is required.[7]

For quantitative determination of testosterone, high-performance liquid chromatography (HPLC),[8, 9] liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS),[10-13] and gas chromatography coupled with mass spectrometric detection (GC/MS) are generally employed.[14] These methods require bulky and expensive instruments, well trained personnel, and multi-step processes such as hydrolysis, extraction, and purification. In addition to chromatographic methods, immunoassays, such as enzyme-linked immunosorbent assay (ELISA), have also been developed for cost-effective, highly-sensitive and specific detection of testosterone in human sera with a simple pre-treatment.[15-17] Mitchell et al,[18] demonstrated an antigen-bound enzyme immunoassay (EIA) with a linker conjugate, to achieve accurate detection of testosterone in saliva samples. Huang et al,[19] developed a competitive immunoassay using microchip electrophoresis combined with chemiluminescence detection for assaying testosterone. However, a straightforward, easy-to-operate and multiple immunoassay for detecting testosterone in both serum and urine samples is still lacking.

Microfluidics-based immunoassays have shown great promise in improving the performance of conventional immunoassays. The operation of microfluidics-based immunoassays is straightforward and flexible. The serum or urine samples do not require complicated sample pre-treatment for microfluidics-based immunoassays, which enable a rapid and sensitive detection of multiple samples inside a small chip. The customized instrument for microfluidic systems may allow for the automated operation and data analysis. Our group previously proposed a variety of prototypes of microfluidic immunoassay chips, such as a vacuum-accelerated microfluidic immunoassay (VAMI) system, and an integrated microchip for HIV antibody detection.[20-24] In this work, we present a microfluidics-based indirect competitive chemiluminescence enzyme immunoassay (MIC) for the multiple detection of testosterone in both serum and urine samples, with improved sensitive and flexibility.

Results and discussion

Testosterone is a hapten with a low molecular weight, and thus difficult to be detected by traditional ELISA that uses immobilized antibody to capture antigen. In contrast, the indirect competitive enzyme immunoassay could be adopted for quantitative assays of

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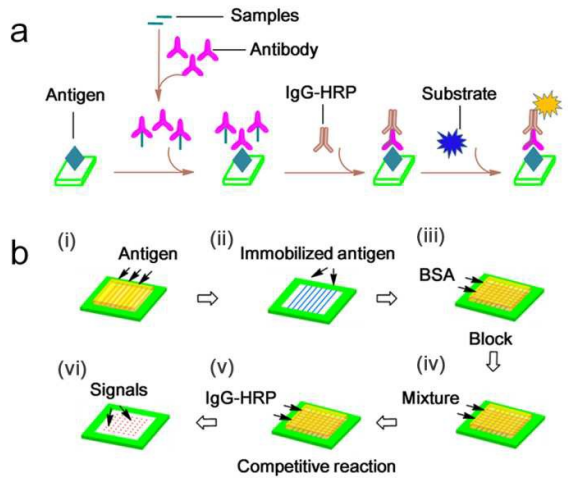

Fig. 1(a) The illustration of indirect competitive reaction for assaying testosterone. (b) Schematic of MIC for detection of testosterone. (i-ii) Introduction and immobilization of antigen T-BSA onto the PDMS substrate via a top PDMS layer; (iii) Block the nonspecific binding sites with 3% BSA; (iv) Indirect competitive reaction by introduction of testosterone sample and T-mAb via a top PDMS layer perpendicular to the immobilized antigen strips; (v) Introduction of the second antibody IgG-HRP; (vi) Introduction of the chemiluminescent substrate to obtain the signal spots of immunoassays.

hapten and other small molecules, which immobilizes hapten-specific capture antigen onto the substrate.[25-26] Unlike the direct immunoassay, the signal of MIC decreases with the increased concentration of hapten. A common protocol for MIC is as follows (Fig. 1a): antigen is coated onto the solid substrate, followed by the introduction of hapten and antibody. The hapten in solution and antigen immobilized on the substrate compete for the binding site of antibody. After the competitive immune reaction, the antibody bound with hapten is washed off by PBST (0.5% tween-20 in phosphate buffer). In contrast, the antibody captured by antigen stays on the substrate. After introducing HRP-conjugated second antibody, the chemiluminescence substrate composed of H_2O_2 and luminol is added for signal generation. HPR can catalyze the H_2O_2 to decompose into H_2O and oxygen free radical, which oxidizes the luminol to yield a luminescence.

The microfluidic chip for MIC consists of two polydimethylsiloxane (PDMS) layers, in which the top layer contains seven parallel microchannels for sample introduction, and the bottom layer is immobilized with antigens (Supporting Information). The procedure of MIC for detection of testosterone involves six steps (Fig. 1b). (1) Testosterone-bovine serum albumin conjugate (T-BSA, 20 µl for each channel) was introduced into the microfluidic channels, and incubated for 30 min to immobilize onto the bottom substrate of PDMS (Fig. 1b(i)). (2) The PDMS substrate was washed with PBS (20 µl) to remove the excess antigen of T-BSA (Fig. 1b(ii)); (3) After immobilization of T-BSA on the substrate, the top layer was peeled off, and a new PDMS top layer was placed with the channels perpendicularly to the immobilized antigen strips on the substrate. Nonspecific binding sites were blocked by introducing 3% bovine serum albumin (BSA, 20 µl) into the microchannels, followed by incubation of 30 min (Fig. 1b(iii)); (4) The mixture of testosterone-mAb (T-mAb, 20 µl) and serum/urine sample was

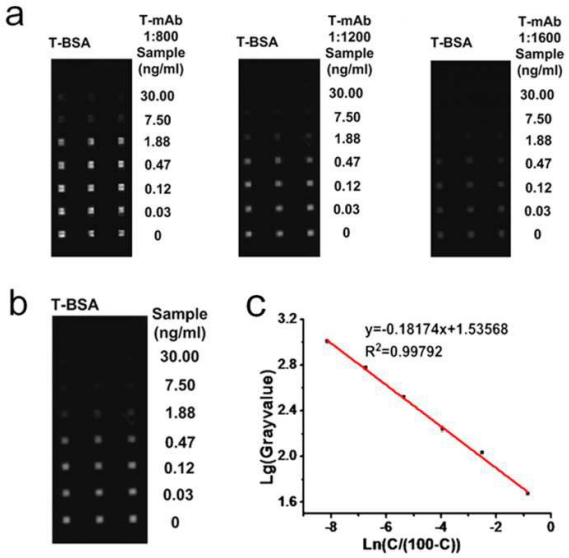

Fig. 2(a) Optimization of T-mAb concentrations (2 mg/ml) with the dilution factor ranging from 800 to 1600. The concentration of T-BSA is 5 µg/ml. Sample is standard testosterone with different concentrations from 0.03 to 30 ng/ml. (b) Indirect competitive reaction for testosterone detection using the microfluidic chip. The chemiluminescent signal spots of testosterone at different concentrations from 0.03 to 30 ng/ml. (c) Standard curve of testosterone versus signal intensity. C is the testosterone concentration. The concentrations of T-BSA, T-mAb, and IgG-HRP are 5.0 µg/ml, 1.67 µg/ml, and 5.0 µg/ml, respectively.

added into the chip. The testosterone in solution and T-BSA immobilized on the substrate competed for the binding site of T-mAb (Fig. 1b(iv)). After a 20-min indirect competitive reaction, the substrate was washed by phosphate buffered saline with tween (PBST, 20 µl) for three times using the microchannels. (5) horseradish peroxidase-conjugated goat anti-mouse IgG (IgG-HRP, the second antibody, 20 µl) was introduced inside the microchannels to incubate for 20 min and the unbound IgG-HRP was removed by PBST washing (Fig. 1b(v)). (6) The chemiluminescent substrate (20 µl) was injected into the chip (Fig.1b(vi)). The chemiluminescence images were captured using a customized instrument (Beijing, China), and the images were analyzed by Image J software (National Institutes of Health). Based on the chemiluminescence intensity, we could calculate the concentration of serous and urinary testosterone by comparing with the calibration curve.

To construct the MIC for testosterone detection in both serum (0.2-7.32 ng/ml) and urine (5-60 ng/ml) samples,[10, 27] several factors that can affect the detection results, including the concentrations of antigen T-BSA, antibody T-mAb, HRP-conjugated second antibody, and incubating time for hapten and antibody binding, have been carefully evaluated. We first optimized the concentration of antigen (T-BSA) by varying the T-BSA concentrations. The T-BSA solutions with three different concentrations of 2.5, 5, and 10 µg/ml were introduced into the multichannel chip, with each concentration having two parallel tests. The antigen solutions of 20 µl for each channel were incubated for 30 min. After immobilization of T-BSA, 20 µl mixtures of T-mAb and testosterone were introduced into the

channels. In the mixture, the concentration of T-mAb was 1.67 μg/ml and that of testosterone was from 0.03 to 30 ng/ml. After the indirect competitive reaction in microfluidic channels, the images were obtained using the customized instrument (Fig. S1). As expected, with the increased sample concentration, the intensity of luminescent signal spot decreased from bright to dark. The intensity of signal spot was increased with increasing the T-BSA concentration from 2.5 to 10 μg/mL. For the T-BSA concentration at 5.0 μg/ml, a good linearity for testosterone measurement at different testosterone concentrations was obtained (Fig. S2). We thus used this concentration for the following experiments.

We next optimized the concentration of T-mAb for MIC with the T-BSA at 5.0 μg/ml. T-mAb (2 mg/ml) solution was diluted by 3 % BSA in PBS solution from 1:1600 to 1:800. After immobilization of T-BSA onto the PDMS substrate for 30 min, we pelt off the top PDMS layer, and placed another clean PDMS top layer perpendicular to the original one. 3 % BSA was introduced into the channels of the top layer, and blocked for 30 min. Mixtures of testosterone and T-mAb (with volume ratio of 1:1, total volume of 20 μl for each channel) were introduced into the chip via top channels, and incubated for 20 min. After removing the mixtures and washing the channels, an excess amount of the second antibody was injected to terminate the immune reaction. The signal spots were developed by adding the chemiluminescence substrate into the channels (Figs. 2a and S3). For the dilution factor of 1600 or 800, the intensity of signal spots was either too dark or too bright, resulting in a poor linear relationship of testosterone at different concentrations. As to the dilution factor of 1200, a good linearity was obtained (Fig. S3). Therefore we used the dilution factor of 1200, corresponding to the T-mAb concentration of 1.67 μg/ml, for the following MIC assays.

With the optimized T-BSA concentration of 5.0 μg/ml and the T-mAb dilution factor of 1200 (1.67 μg/ml), we tested the concentrations of second antibody with a dilution factor from 200 to 800. After the immobilization of T-BSA, the binding site of T-mAb in solution was competitively occupied by testosterone in solution and immobilized T-BSA. By washing off the conjugation of T-mAb and testosterone, the second antibody, IgG-HRP of 20 μl, was introduced into the microchannels to react with the captured T-mAb by T-BSA for 20 min. The dilution factors of IgG-HRP (2 mg/ml) were 200, 400, and 800. After the indirect competitive reaction, the assaying results were recorded (Fig. S4). Similar to the previous optimization process, we found that a dilution factor of 400 yielded a good linearity (Fig. S5). The incubating time for hapten and antibody binding was also optimized. After the immobilization of T-BSA, the mixtures of testosterone and T-mAb (with volume ratio of 1:1, total volume of 20 μl for each channel) were introduced into the chip via top channels, and incubated for 20 min or 30 min. There is no significant difference of immunoassay signals after different incubating times (Figs. S6 and S7), and we chose 20 min to shorten the total assay time.

After the optimization of concentrations of T-BSA (5.0 μg/ml), T-mAb (1.67 μg/ml), and IgG-HRP (5 μg/ml), we produced the standard curve of testosterone at different concentrations from 0.03 to 30 ng/ml. As expected, the increased concentration of testosterone in mixture will lead to the less T-mAb captured by the immobilized T-BSA, resulting in the decreased intensity of chemiluminescent signals (Figs. 2b). We found a strong linear relationship between Ln(C/(100-C)) and Lg(Grayvalue), in which C is the testosterone concentration from 0.03 to 30 ng/ml, and Gray value is the recorded signal intensity (Y=1.53568-0.18174X; R^2= 0.99792). From the standard curve in Fig. 2c, the limit of detection (LOD) for the testosterone assay was determined by: LOD=3σ/a, in which σ is the slope of the equation; σ is the standard deviation from three repeats. The LOD of testosterone detection by MIC is calculated to be 0.02 ng/ml.

To demonstrate the clinical applications of MIC, we tested the levels of testosterone in 49 human serum samples, and compared the results with two commercial testosterone kits (Beifang and Biocheck) (Fig. 3). The serous testosterone concentrations measured by MIC were in good agreement with those obtained using Biocheck's ELISA kit (the linear range: 0.1-18 ng/ml, and LOD: 0.05 ng/ml) with the following equation: Y =0.8565X+0.2616, and R^2 = 0.9561, while the comparison between MIC and Beifang chemiluminescence kit (the linear range: 0.2-25 ng/ml, and LOD:0.1 ng/ml) yielded the equation of Y =1.1858X+0.4977, and R^2 = 0.8976. We should note that the two commercial kits showed fairly different results for the same serum sample, possibly due to the different immunoassay methods: ELISA kit from Beifang is based on direct competitive immunoassay, and the kit from Biocheck is based on indirect competitive immunoassay. The linear fit between two commercial kits was: Y=0.5055X+0.3273 and R^2 = 0.8613 (Fig. S8). Since the MIC was also based on indirect competitive immunoassay, its results were more comparable to Biocheck kit than Beifang kit.

Coefficients of variation(CV) for evaluation of the precision and reproducibility of MIC was determined by the following formula: $CV=(\sigma/MN)\times 100\%$, in which σ is the standard deviation, and MN is the mean value of signal intensity. The same sample was assayed repeatedly for 42 times to determine the CV of within-chip measurements and twice each day for 11 consecutive days to obtain the CV of between-chip measurements. The CV was 7.99 % for within-chip and 12.43 % for between-chip assays.

To determine the accuracy of MIC, different amounts of testosterone in 3% BSA, serum and urine were analyzed by MIC and ELISA. The measurement results are summarized in Table 1. The relative errors (RE) between ELISA values and MIC values were in the range of ±10% for three different kinds of samples (Supporting Information). MIC could thus be employed for testosterone detection in both serum and urine with high accuracy, reliability and small interference.

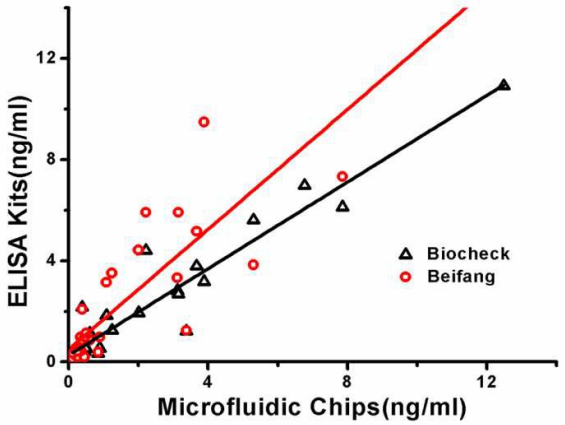

Fig. 3 Testosterone measurement in 49 human serum samples. Each serum sample is measured by MIC, Biocheck's and Beifang's ELISA kit, to compare the performance of MIC with commercial kits.

Conclusions

In summary, a microfluidics-based multiple, sensitivity, and indirect competitive chemiluminescence enzyme immunoassay (MIC)for the determination of testosterone was presented. The linear range of MIC is from 0.03 to 30 ng/ml, the LOD is 20 pg/ml, and the average recovery is within 110%. For testosterone detection, MIC shows a good correlation with the commercial kits. The advantages of MIC include: (1) MIC is easy-to-operate and portable, thus not requiring large equipment or skilled personnel. (2) MIC is a rapid immunoassay. The total reaction time is less than 40 min, while commercial kits requires a total assay time of 60 min. (3) MIC can simultaneously detect seven samples on a small chip. (4) MIC dramatically reduces the consumption of sample and reagents with a total amount of 40 μl, in comparison with 200 μl for commercial kits. (5) MIC is operated at room temperature, while the assay temperature for commercial kits is 37°C. Therefore, we believe that this technique may have broad applications for clinical practices.

Table 1 Results for determination of testosterone in different samples.

Solution	Testosterone content	ELISA (ng/ml)	MIC (ng/ml)	RE (%)[a]
Serum	High	10.00	10.17	1.70
	Low	1.00	1.07	7.00
Urine	High	10.32	11.00	6.59
	Low	5.49	4.99	-9.11
3% BSA	High	10.00	9.75	-2.50
	Low	1.00	0.95	-5.00

[a] Relative errors(RE)=(MIC value- ELISA value)/ELISA value×100%

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