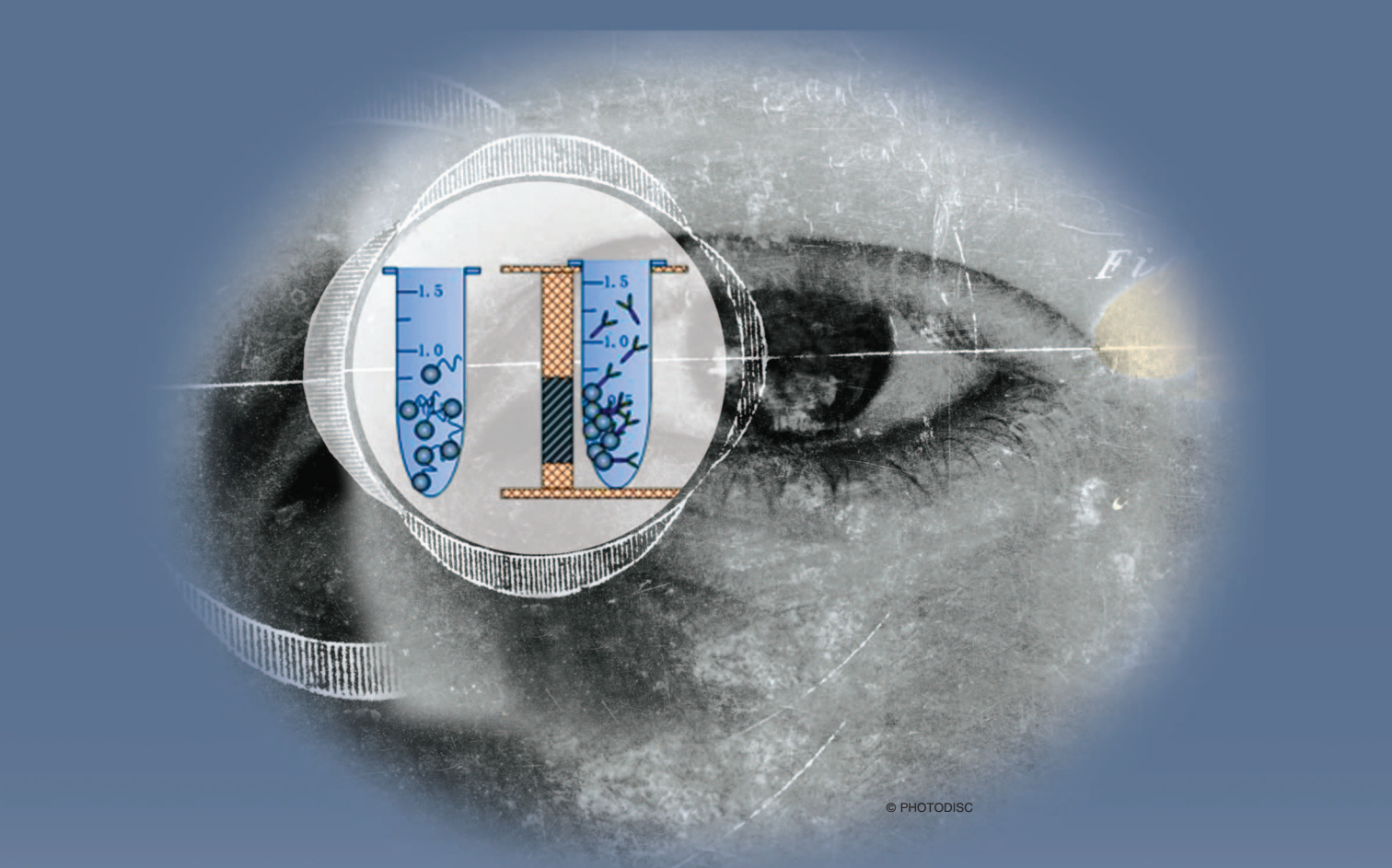

Insulin Detection of Clinical Plasma Samples

Development of a PDMS microfluidic system.

A POLYDIMETHYSILOXANE (PDMS)-based microfluidic system with pneumatic micropumps and micromixers has been developed for high-accuracy insulin detection of clinical plasma samples. The detection method is based on double-antibody sandwich immunoassay coupled with luminol-hydrogen peroxide (H_2O_2) chemiluminescence.

Diabetes mellitus is a group of diverse metabolic disorders caused by deficiency or diminished effectiveness of endogenous insulin [1]. It is the third leading cause of disease-related death around the world after cancer and cardiovascular disease. At present, the blood glucose test is the most popular clinical diagnostic tool for diabetes, but irregular blood glucose level, unfortunately, is not a good early indicator for diabetes diagnosis. Previous studies have shown that insulin, connecting peptide, and interleukin-6 are better suited as early diagnostic immune indicators for the onset of diabetes [2]. Insulin, a hormone produced by the pancreas for converting glucose to energy, is a common clinical indicator. Its abnormity can be detected as an

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effective auspice to diagnose diabetes at an early stage, which is helpful for diabetes prevention. The traditional test methods for insulin and other indicators in clinical diagnosis, which include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, and various chemiluminescence-based techniques, are usually based on microplates and large automatic biochemistry analyzers. These methods, while effective, are predominantly laboratory based and require trained technicians. This makes them unsuitable for field testing, which is becoming increasingly important for diabetes screening in developing countries.

Microfluidics, with advantages such as low reagent consumption, short analysis time, high reliability and sensitivity, and multiprocess integration [3], [4], is a rapidly developing field in biomedical testing [5]. In microfluidic systems, the conventional biochemical processes, such as sample pretreatment, sample/reagent transport, mixing, reaction, separation, detection, and product collection, can be carried out automatically on a single chip [6]. Additionally, microfluidic systems have become the most promising tool for handling costly and difficult-to-obtain samples and reagents [7]. More importantly, the portability characteristic of these systems that arises from their compact form is a key factor for point-of-care applications [6] and offers a practical solution to diabetes screening in a rural environment.

Recently, several advancements in measuring insulin using microfluidic devices coupled with novel detection techniques were demonstrated. A carbon nanotube/dihydropyran composite sensor in a microfluidic device has been reported to have a detection limit of 1 μM [8]. A SlipChip-based approach was demonstrated to perform bead-based heterogeneous immunoassays with multiple nanoliter-volume samples. An insulin immunoenzymatic assay showed a detection limit of about 13 pM [9]. Another approach used a microfluidic local-surface plasmon resonance chip operated with a simple collinear optical system to interrogate specific insulin and antiinsulin antibody reaction in real time and demonstrated a detection limit of 100 ng/mL insulin [10]. Furthermore, by conjugating with a continuous-flow immunosensor, the detection limit of the surface plasmon resonance (SPR) method can be improved to 1 ng/mL (ppb) with a response time of less than 5 min [11]. However, these methods still require labor-intensive processing, large equipment, and trained experts to provide accurate insulin measurements.

> Diabetes mellitus is a group of diverse metabolic disorders caused by deficiency or diminished effectiveness of endogenous insulin.

In the present study, an integrated PDMS-based microfluidic system coupled with magnetic bead-based sandwich ELISA has been developed to measure insulin concentrations. Superparamagnetic beads coated with insulin antibodies are used to capture the target insulin molecules. The integrated microfluidic system that is composed of two pneumatic micropumps and a micromixer automates the immunoreaction process. A chemiluminescent detector is then used to quantify the concentration of the insulin captured by the immunobeads in the micromixer. Using this approach, the developed microfluidic system achieves a low detection limit and a short reaction time.

MATERIALS AND METHODS

Figure 1 shows a schematic illustration of the experimental procedure for insulin detection using heterogeneous chemiluminescent immunoassays, which are typically performed manually in biomedical laboratories. In this procedure, carboxyl-modified magnetic microspheres with a mean diameter of approximately 5 μm are used as solid-phase supports to covalently conjunct primary insulin antibodies in centrifuge tubes [Figure 1(a)]. The immunocompetent antibodies conjugated on the bead surface allow the beads to capture the target insulin (antigen). Taking advantage of the specific interaction between the immune beads and insulin, the target insulin molecules are then recognized and form a half sandwich structure in the micromixer chamber with the microbeads. In the presence of an external magnet, the nonreactive insulin molecules are washed away [Figure 1(b) and (c)]. Then the insulin antibodies labeled with horseradish peroxidase (HRP) are added to form complete sandwich complexes [Figure 1(d) and (e)].

After removing the nonreactive materials, luminal and H_2O_2 with the enhancer *p*-iodophenol are injected. Under the condition of alkalescence and catalyzed by the HRP of the sandwich complexes, the injected luminol is rapidly oxidized by H_2O_2 and emits light at a wavelength of approximately 425 nm with an intensity proportional to the quantity of HRP [Figure 1(f)]. Since the insulin and its secondary antibody are a one-to-one conjugation, the quantity of HRP connected to the secondary Ab is a direct indication of the quantity of insulin. The emitted light is quantified by a luminometer. All insulin-related bioreagents are obtained from Shanghai E. Star BioTechnology Co.

The immune-related processes are carried out semiautomatically in the microfluidic system. Traditionally, the immune magnetic beads and biosamples are first incubated for 60 min to recognize the target insulin. After incubation, the target insulin adhered onto the magnetic beads is captured using an external magnet. Then, a wash buffer [phosphate buffered saline Tween-20 (PBST)] is added to wash away the nonreactive materials. The HRP-labeled insulin antibody is then manually added and allowed to interact with the target insulin for 30 min. The unbound, HRP-labeled insulin antibody is then washed away. Finally, luminol, H_2O_2, and the enhancer (*p*-iodophenol) are added for chemiluminescence detection using a luminometer.

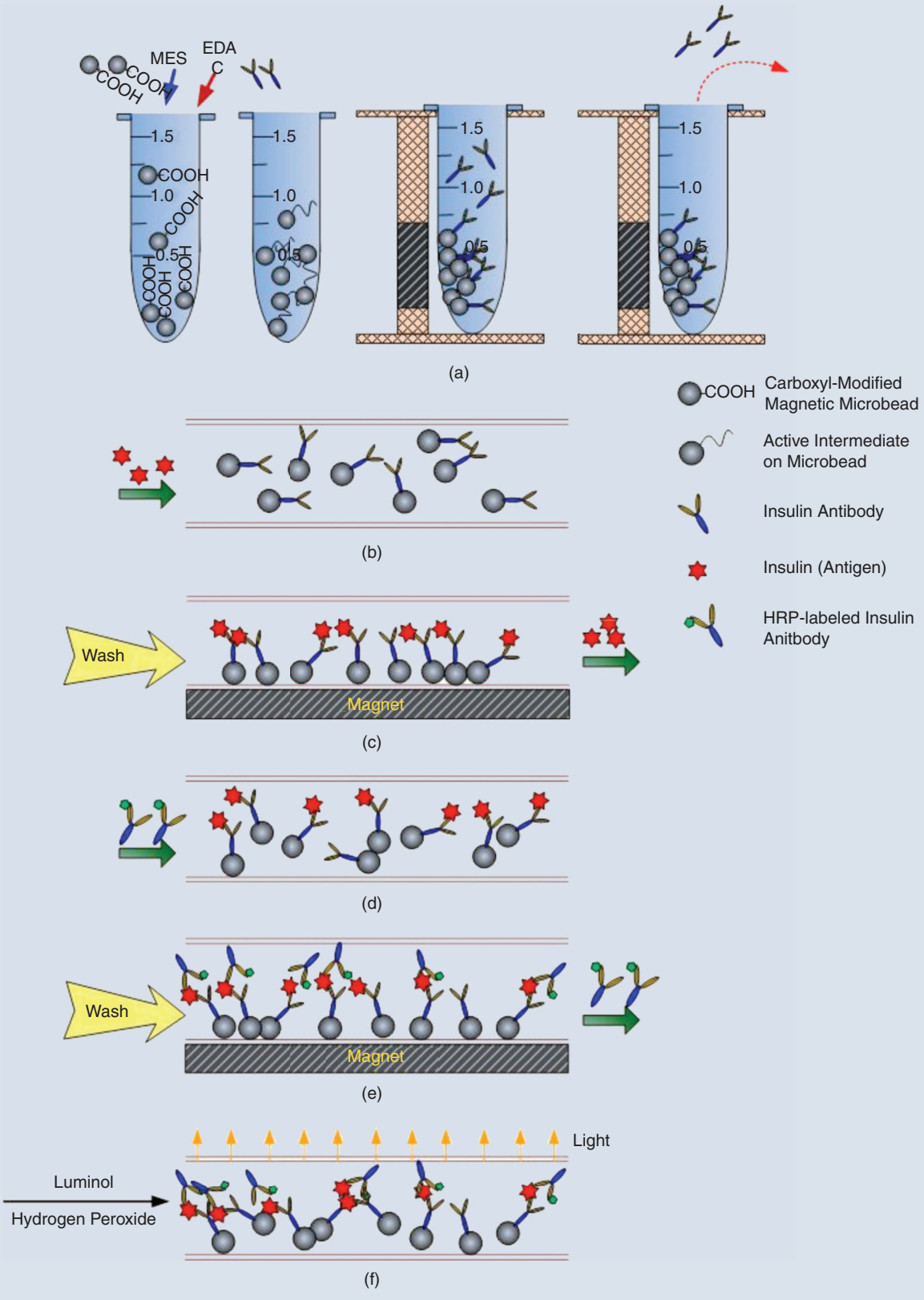


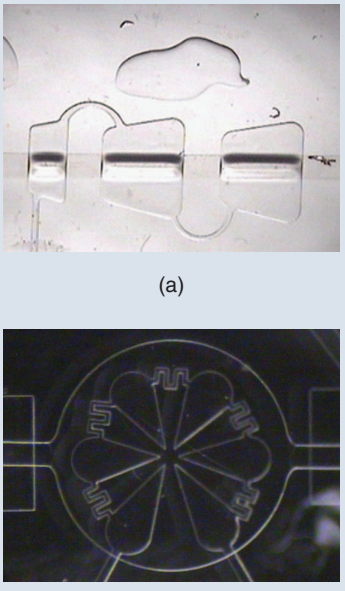
FIGURE 1 Experimental procedure for insulin detection using heterogeneous chemiluminescent immunoassays.

MICROFLUIDIC SYSTEM

The integrated microfluidic system is composed of two PDMS-based pneumatic micropumps and a micromixer. Tygon tubes connect the outlets of the micropumps to the inlets of the micromixer, as shown in Figure 2.

The PDMS-based pneumatic micropump and micromixer have similar structures (Figure 3) and working principles. Both designs consist of two thin PDMS layers. The top layer contains either a microchannel (micropump) or a mixing chamber (micromixer) with either an inlet and an outlet or two y-shaped inlets and an outlet. The height of the microchannel is 100 μm while the diameter and depth of the mixing chamber are 2 mm and 100 μm, respectively. The micropump and micromixer are operated based on pneumatic peristaltic principles. Their bottom PDMS layer consists of either three (micropump) or six (micromixer) membrane-enclosed air chambers. In the micropump, the three serially connected air chambers have different volumes and generate fluid movement in the microchannel through a sequential diaphragm deflection [12]. In the micromixer, the six air chambers have identical volumes. Mixing is achieved by pneumatically deflecting the six 100 μm-thick diaphragms of the air chambers. The chambers are connected in series to each other through serpentine microchannels. A miniature air compressor is used to supply the required compressed air and is controlled by an electromagnetic valve (EMV). Details of the fabrication process flow for the micropump and micromixer can be found in [13].

> Microfluidics is a rapidly developing field in biomedical testing.



(a)

(b)

FIGURE 2 (a) PDMS micropump and (b) micromixer.

EXPERIMENTAL RESULTS AND DISCUSSIONS

EXPERIMENTAL SETUP

Figure 4 demonstrates the experimental setup of the insulin detection scheme. The setup consists of a mini air compressor and a custom-designed circuit that controls the opening and closing of EMV to control the air flow into the micropump and micromixer. A pressure sensor is utilized to monitor the air pressure. The chemiluminescence signal from the micromixer is detected by a photometer (RFL-1A).

CHARACTERIZATIONS OF THE PNEUMATIC MICROPUMP AND MICROMIXER

The micropump and micromixer are extensively tested before insulin detection to determine their operating parameters. The effects of the applied air pressure and the EMV driving frequency on the resultant flow rate of the micropump are explored using ultra pure water. As shown in Figure 5, the general trend of the flow rate under a constant pneumatic pressure is ascending with the increasing driving frequency.

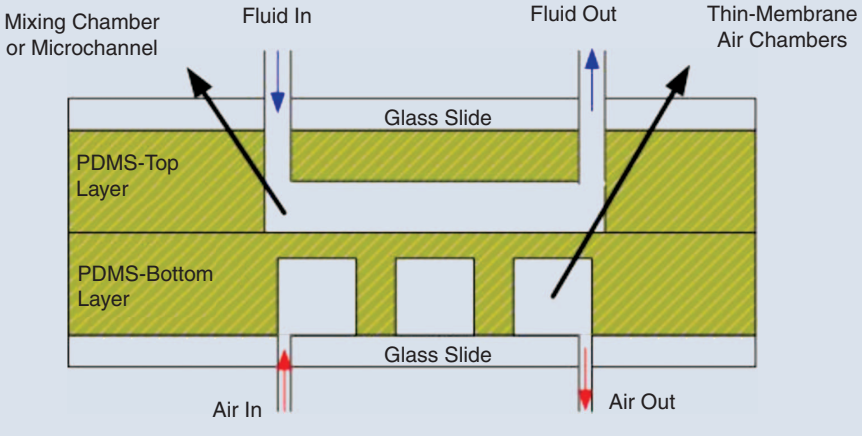


FIGURE 3 Cross-sectional view of the PDMS micromixer/micropump.

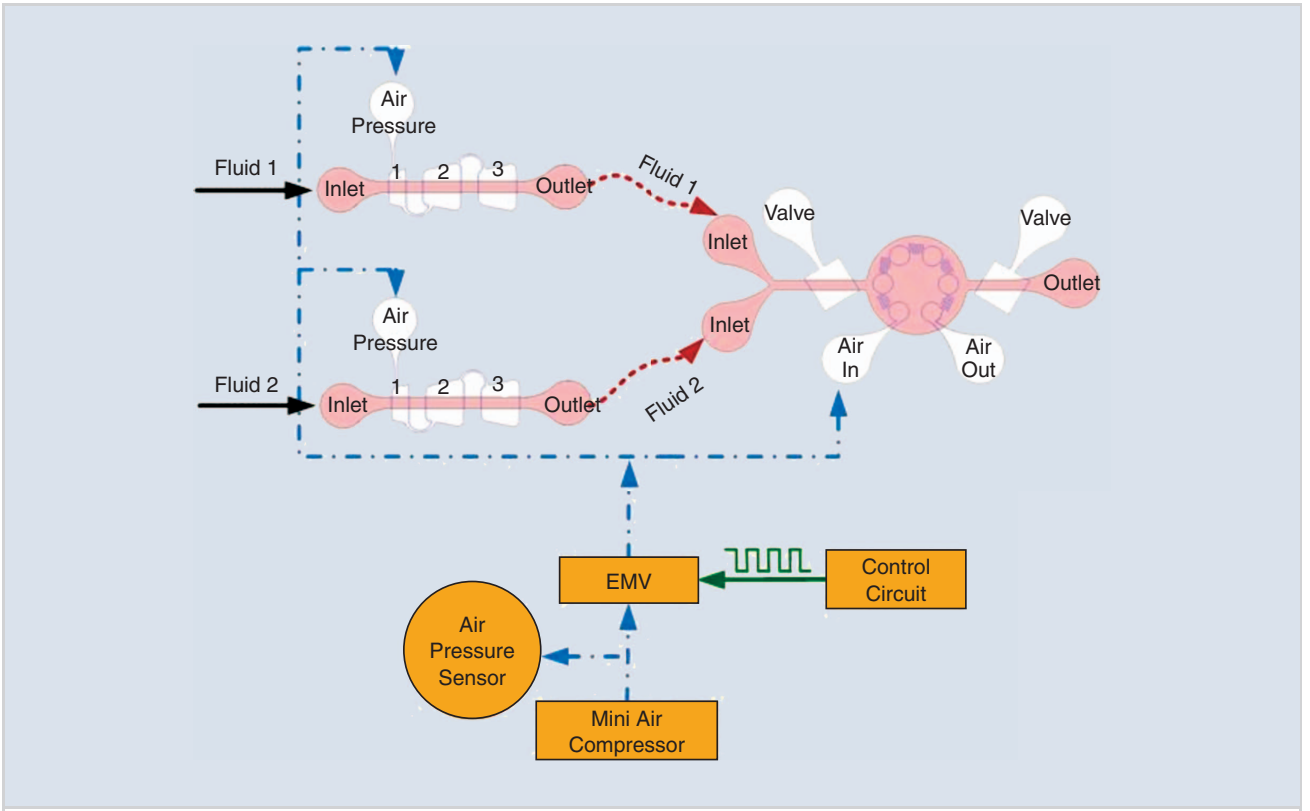


FIGURE 4 Integrated microfluidic system with two pneumatic PDMS micropumps and a micromixer.

However, the maximum achievable flow rate is limited by the speed of the filling and emptying of the air chambers. When the EMV frequency is too high, the PDMS diaphragm cannot completely deflect and recover back to its original state. This causes the flow rate to fall after an optimal frequency is reached.

The micromixer's performance is also evaluated under different applied air pressures and EMV driving frequencies. The peak chemiluminescent light intensity when the reagents flow through the mixing chamber at a constant flow rate of 25 μL/min is used as a measure of the mixing efficiency. The chemiluminescent reagent concentration used is 4.8×10^{-3} M for luminol and 7.5×10^{-3} M for H_2O_2. The result indicates that at 5 and 10 lbf/in^2, the peak chemiluminescent intensity increases with the increasing frequency up to 10 Hz. At 15 lbf/in^2, the light intensity decreases with the increase in frequency. On the basis of this result, it is determined that the optimal working parameters of the micromixer will be 10 lbf/in^2 and 5 Hz.

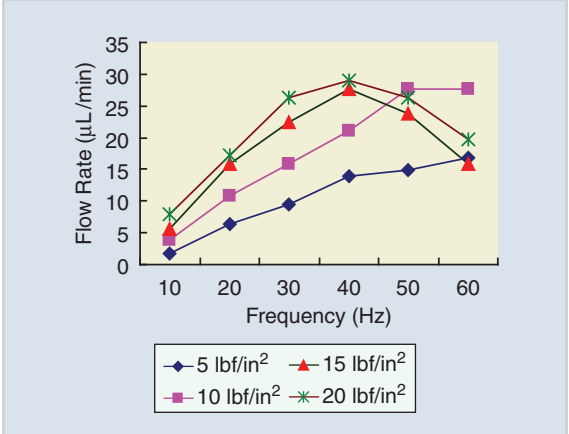


FIGURE 5 Flow rates of the pneumatic micropump under different operating parameters.

INSULIN MEASUREMENT

The serum samples used in the present study were obtained from diabetic patients at the Chinese Medical University Hospital, Shenyang, China. Since the insulin concentration is almost the same as our detection limit of 10^{-10} M, the serum sample is pretreated by adding nonclinical insulin to make higher concentration solutions. The nonclinical insulin is purchased from Shanghai E. Star BioTechnology Co.

Nonclinical insulin is diluted by phosphate buffered saline (PBS) (pH 7.4) to be 9.5×10^{-7} M, 4.75×10^{-8} M, and 4.75×10^{-9} M. The insulin original concentrations in clinical postprandial serums of three different patients are measured by a biochemical analyzer. They are 68.94, 42.25, and 37.53 mIU/L, respectively, but become 4.75×10^{-7} M, 2.39×10^{-8}, and 5.0×10^{-9} M after adding nonclinical insulin.

The insulin measurement process is divided into two procedures. The immunoreactions and the related washing steps are carried out in the integrated

microfluidic system shown in Figure 2. The chemiluminescent reaction is accomplished in a separate micromixer with the help of a syringe pump for reagent injection. For the microfluidic system, the biochemical reagents and wash buffers are delivered into the mixing chamber by two micropumps operated at an almost identical pumping rate of approximately 11 μL/min. The mixer is operated at a driving frequency of 5 Hz and an applied air pressure of 10 lbf/in^2.

The immunoreaction process begins with the transport of conjugated magnetic beads produced from the process described in Figure 1(a) and the insulin solution into the mixing chamber to form half-sandwich structures [Figure 1(b) and (c)]. Then, these structures and the HRP-labeled insulin antibodies are brought together to form the sandwich complex [Figure 1(d) and (e)] for the subsequent chemiluminescent reaction. Washing procedures are carried out following each immune reaction with the help of an external permanent magnet.

The micromixer used for the chemiluminescence detection is placed in the dark box of the photometer to reduce noise and improve sensitivity. A double-channel syringe pump is used to inject reagents into the mixing chamber via a Y-shaped microchannel. The solution containing the HRP-labeled insulin sandwich complex, hydrogen oxide (7.5×10^{-3} M), and the enhancer p-iodophenol (3 mg/mL) is injected by one syringe while a second syringe is used to deliver the luminol solution (7.8×10^{-3} M). The two solutions are allowed to blend well and react [Figure 1(f)].

The three samples with different insulin concentrations are examined four times within a single day and repeated three times every two days. Figure 6 demonstrates the average sensitivity curve. Overall, the result indicates that the detection limit is 10^{-11} M with a linear range of 5×10^{-10} M–6.4×10^{-9} M. The repeatability test results indicate an average coefficient of variation (CV) value of 14.95% for samples in the same batch and 15.70% for different batches. This implies that the insulin detection method investigated in this article is more consistent for samples within the same batch. The detection time of the microfluidic system is about 10 min.

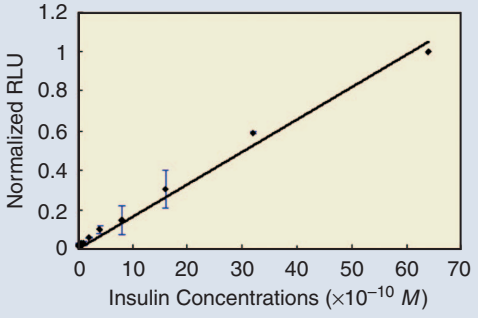


FIGURE 6 Relationship between insulin concentration and the corresponding chemiluminescence signal relative luminescence units (RLUs).

CONCLUSIONS

The current study demonstrates the application of a PDMS microfluidic system for insulin detection of clinical plasma samples based on heterogeneous chemiluminescent immunoassays. The detection method utilizes two pneumatic micropumps operated at a flow rate of about 10 μL/min and a micromixer operated at a frequency of 5 Hz. The detection limit of the PDMS microfluidic system is 10^{-11} M with a linear range of 5×10^{-10} M – 6.4×10^{-9} M. The detection time is about 10 min, which is more than ten times faster than that of the conventional methods for insulin detection.

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