



Multiplex detection of tumor markers with photonic suspension array

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ABSTRACT

A novel photonic suspension array was developed for multiplex immunoassay. The carriers of this array were silica colloidal crystal beads (SCCBs). The codes of these carriers are the characteristic reflection peak originated from their structural periodicity, and therefore they do not suffer from fading, bleaching, quenching, and chemical instability. In addition, because no dyes or materials related with fluorescence are included, the fluorescence background of SCCBs is very low. With a sandwich format, the proposed suspension array was used for simultaneous multiplex detection of tumor markers in one test tube. The results showed that the four tumor markers, α -fetoprotein (AFP), carcinoembryonic antigen (CEA), carcinoma antigen 125 (CA 125) and carcinoma antigen 19-9 (CA 19-9) could be assayed in the ranges of $1.0\text{--}500\text{ ng mL}^{-1}$, $1.0\text{--}500\text{ ng mL}^{-1}$, $1.0\text{--}500\text{ U mL}^{-1}$ and $3.0\text{--}500\text{ U mL}^{-1}$ with limits of detection of 0.68 ng mL^{-1} , 0.95 ng mL^{-1} , 0.99 U mL^{-1} and 2.30 U mL^{-1} at 3σ , respectively. The proposed array showed acceptable accuracy, detection reproducibility, storage stability and the results obtained were in acceptable agreement with those from parallel single-analyte test of practical clinical sera. This technique provides a new strategy for low cost, automated, and simultaneous multiplex immunoassay.

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1. Introduction

With more than 10 million new cases and 7.6 million deaths every year, cancer has become one of the most devastating diseases worldwide [1]. Yet, many of these deaths can be avoided by early detecting, treating and curing. The measurement of tumor markers has been showing its significance in early screening of cancer, differentiating benign from malignant conditions, evaluating the extent of disease, monitoring the response of cancer therapy, and predicting recurrence [2,3]. However, a single tumor marker is usually not sufficient to diagnose cancer due to its limited specificity. Thus, multiplex immunoassay of tumor markers has attracted considerable interest to meet the growing demand for diagnostic application [4–6]. Furthermore, multiplex immunoassay can offer higher sample throughput, less sample consumption, shorter assay time and lower cost than the traditional parallel single-analyte immunoassay [7].

Up to now, various assay approaches have been devised to realize simultaneous multiplex analysis. Most of them are based on molecular binding or recognition events. In order to distinguish

different binding events in parallel, molecules should be encoded. Biochip, in which a large number of different probe molecules are immobilized on a flat substrate and encoded by the coordinate of their positions on a two-dimensional grid, had led to a dramatic development in the past 20 years. However, biochips still have certain drawbacks, including slow diffusion of molecules to their binding sites and the inability to perform large numbers of reactions simultaneously over a wide dynamic range [8,9]. Recently, suspension arrays, in which probes are attached to the surface of microparticles, become an attractive alternation for multiplex analysis [10–12]. Such arrays offer higher flexibility for detecting new analytes and show faster reaction kinetics in solution due to the radial diffusion of analytes or probes [13]. Among various suspension arrays, those arrays with spectrum-encoded microparticles are well used due to their simplicity in both encoding and detection. Fluorescent dyes [14–16] and quantum dots [17–20] are the main spectrum-encoding elements and the microparticles encoded by fluorescence have been commercialized by Luminex and some other companies [12]. However, the fluorescence dyes tend to be quenched or bleached and the quantum dots are usually biotoxic [21,22]. Moreover, the fluorescence of the carriers can interfere with the signal from the labeling molecules and as a result affect the detection limit. In this point of view, we proposed the silica colloidal crystals beads (SCCBs) as encoded supports for suspension array [23,24]. Code of SCCBs is the characteristic reflection peak originated from the stop-band of colloid crystal [25]. As the peak

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position is based on its periodical structure, the code is very stable and the fluorescent background is low. In addition, the SCCBs provide much higher surface-to-volume ratio which means more dye molecules participate in photon absorption and subsequent photon emission. These properties make the photonic suspension array suitable for high sensitive detection.

In this paper, we designed a photonic suspension array based on SCCBs for multiplex detection of tumor makers. The use of this array could not only increase the detection sensitivity but also simplify the decoding and bio-reaction detection process. With a sandwich format, the proposed suspension array was used for multiplex immunoassay of four tumor markers, α -fetoprotein (AFP), carcinoembryonic antigen (CEA), carcinoma antigen 125 (CA 125) and carcinoma antigen 19-9 (CA 19-9), which show great significance in early screening and clinical diagnosis of some tumor diseases including hepatocellular cancer, yolk sac cancer, colorectal cancer, gastric cancer, and lung cancer [26–28]. The results obtained were in acceptable agreement with those from the parallel single-analyte test of practical clinical sera. The photonic suspension array possesses attractive characteristics such as low cost, simple manipulation, and easy automation and has the potential to assay more analytes.

2. Experimental

2.1. Materials

Human AFP, CEA, CA125, CA19-9, and Mouse monoclonal anti-human AFP antibody, anti-human CEA antibody, anti-human CA125 antibody, anti-human CA19-9 antibody, and fluorescein isothiocyanate (FITC) tagged goat anti-human CA125 antibody, anti-human CA19-9 antibody and anti-human CEA antibody were obtained from Usclife Co., USA. Bovine serum albumin (BSA) was purchased from Sigma Chemicals. 3-aminopropyltriethoxysilane (APTES) and glutaraldehyde were brought from Alfa Aesar Co. Monodisperse silica nanoparticles were synthesized by Stöber method [29]. Polydimethylsiloxane (KF-96 10 cSt) was gained from Shin-Etsu Chemical, Japan. Clinical serum samples were gifts from Zhongda Hospital, China. Phosphate buffer saline (PBS, 0.05 M, pH 7.4), phosphate buffer saline tween-20 (PBST, 0.05% tween-20 in PBS) and phosphate buffer (PB, 0.05 M, pH 5.0) were self-prepared. All buffers were prepared with water purified in a Milli-Q system (Millipore, Bedford, MA). All other reagents were of the best grade available and used as received.

2.2. Instrumentation

The microfluidic device used for SCCBs generation was home-made. A needle with an inner diameter of 60 μm and outer diameter of 250 μm was inserted into the main inlet of a T-junction [24]. A magnetic pump was used to feed silica nanoparticles aqueous solution as the dispersed phase through the needle. Another inlet of the T-junction was connected to a syringe pump, through which oil used as the continuous phase was fed. The drops generated at the outlet were guided to a container for collection.

The microstructures of SCCBs were characterized by a scanning electron microscopy (SEM, HITACHI, S-300N). Photographs of SCCBs were taken with an optical microscope (OLYMPUS BX51) equipped with a CCD camera (Media Cybernetics Evolution MP 5.0). Reflection spectra of SCCBs were recorded by a microscope equipped with a fiber optic spectrometer (Ocean Optics, USB2000). Fluorescence spectra of SCCBs were recorded by a microscope equipped with a fiber optic spectrometer (Ocean Optics, QE65000).

2.3. Generation of SCCBs

The silica colloidal crystal beads were fabricated by droplet template method [24]. Firstly, the aqueous suspension containing monodisperse silica nanoparticles was broken into droplets by the oil flow in the microfluidic device, and the droplets were taken into the collection container which was also filled with the silicon oil. Then, the silica nanoparticles self-assembled into ordered lattices during the evaporation of water in the droplets at 60 °C. After solidification, the silica colloidal crystal beads were thoroughly washed with hexane to remove the silicon oil. Finally, the silica colloidal crystal beads were calcined at 700 °C for 3 h to improve their mechanical strength. To meet the demand of multiplex immunoassay, four kinds of aqueous suspension containing monodisperse silica nanoparticles with diameters of 195 nm, 247 nm, 264 nm, and 314 nm, respectively, were used for the colloidal crystal beads fabrication. The concentration (w/v) of the four kinds of silica nanoparticles was 15%. The injection speed of oil phase was 15 mL h^{-1} and the injection speed of dispersed phase was 0.5 mL h^{-1} . Finally, four kinds of 180 μm SCCBs with the reflection peak position in 428, 543, 580 and 692 nm, respectively, were generated.

2.4. Probes immobilization

Anti-tumor marker antibody probes were immobilized on SCCBs by covalent bonding method. Firstly, the SCCBs were treated with piranha solution (30% hydrogen peroxide and 70% sulfuric acid) for 6 h. After washed with water and dried by nitrogen flow, the beads were treated with an ethanol solution of APTES (5%) and PB buffer of glutaraldehyde (2.5%) for 4 h in turn. Then, the SCCBs were reacted with anti-tumor marker antibody probes (0.1 mg mL^{-1} , about 0.1 μL per bead) in PBS buffer solution at 4 °C for 12 h. Afterward, the beads were treated with 1 mg mL^{-1} NaCNBH₃ solution for 1 h at 4 °C. Finally, the unreacted groups of glutaraldehyde on the SCCBs' surface were passivated with 1% BSA PBS buffer for 2 h. For multiplexed immunoassays, four kinds of these SCCBs, with the reflection peak position in 428, 543, 580 and 692 nm, were modified with mouse monoclonal anti-human AFP antibody, anti-human CEA antibody, anti-human CA125 antibody, anti-human CA19-9 antibody, respectively. The fraction of immobilized antibodies was assessed by comparing the fluorescence signals produced by fluorescein isothiocyanate (FITC) tagged goat anti-tumor marker antibodies solution before and after immobilization, and the antibody immobilization efficiency was about 12%. For the immobilization of antibody, thousand of beads could be coupled per round in one tube.

2.5. Detection of tumor markers

For single analysis, different concentrations of tumor marker (tumor marker in PBS) were used to incubate anti-tumor marker antibody-modified SCCBs (1 μL per bead) in the test tubes for 30 min, and unbound tumor marker was washed away with 1% BSA PBS buffer. Then, fluorescein isothiocyanate (FITC) tagged goat anti-tumor marker antibody (10 $\mu\text{g mL}^{-1}$) was added to the test tubes and incubated for another 30 min. During all the incubation process, the test tubes were shaken at 37 °C. Fluorescence spectra of SCCBs were measured after thoroughly washing with 1% BSA PBST buffer. The number of replicates at the any concentration was 5. The detection limit was calculated from the zero calibrator plus three times the standard deviation.

For multiplexed detection of tumor markers, four kinds of SCCBs, with the reflection peak position in 428, 543, 580 and 692 nm, immobilized with anti-human AFP antibody, anti-human

CEA antibody, anti-human CA125 antibody, and anti-human CA19-9 antibody, respectively, were put into one test tube. Then the serum sample suspension was added. After being shaken for 30 min at 37 °C and washed with 1% BSA PBS buffer, the SCCBs were incubated in the mixed solution containing the four fluorescein isothiocyanate (FITC) tagged goat anti-tumor marker antibodies. At last, the SCCBs were thoroughly washed with 1% BSA PBST buffer and put into the beads chamber for decoding and bio-reaction detection. The number of replicates at the any serum sample detection was 5.

All the operations of tumor marker detection by electrochemiluminescence immunoassay (ECLIA) were carried out automatically with a MODULAR ANALYTICS E170 (Roche).

3. Results and discussion

3.1. Design of suspension array

Sensitivity and high-throughput application are the two most important advantages of suspension array. Generally, the sensitivity of the suspension array can be affected by the character of the probe carriers, antibodies (monoclonal or polyclonal), and detectors. For our suspension array, the structure of SCCB carriers is the most novel characteristic that may affect on the sensitivity. As the SCCBs are derived from the assembly of monodisperse colloidal nanoparticles in droplet templates, the surface of SCCBs shows ordered hexagonal symmetry of the nanoparticles (Fig. 1). Except providing more surface area for biomolecules immobilization and reaction, this surface structure of SCCBs also provide a nanopatterned platform for immunoreactions. Due to the reduction of the steric hindrance of the molecules via the artificial separation imposed by the nanopattern, the molecules on this platform are free to react with their specific complements and the efficiency of the reaction increases [30]. To primarily implement the high-throughput application of the photonic suspension array, we developed a simple platform by equipping a fiber optic spectrometer to the microscope for the SCCBs decoding and bio-reaction detection. When the SCCBs were exposed to the white light under normal incidence through the microscope, the reflection peaks could be detected, and the abscissa of the peak could be recorded as the peak position for decoding. When the input white light was replaced by blue light with the wavelength at 488 nm, the fluorescence spectra of the SCCBs were recorded for analyzing. This method would be further developed by introducing the SCCBs into flow-through device, such as Flow Cytometer, for the decoding and bio-reaction detection of photonic suspension array.

3.2. Optimization of analytical conditions

In the process of decoding and bio-reaction detection, the size of the SCCBs was the main effects on the suspension system. Too large size of SCCBs would be difficult to be dispersed into the sample solution, while too small size of SCCBs would have high polydispersities due to our limited technique of beads fabrication and thus increase the coefficients of variation (CVs) among repetitions during immunoassays. In this paper, four kinds of the uniform 180 μm multicolor SCCBs (Fig. 2), with their reflection peak position in 428, 543, 580 and 692 nm, respectively, were used for the detection of tumor markers in the following experiments.

In the process of immunoreactions, the incubation time was important for immunoassays. Both the long and short incubation time of immunoreactions would lead to enlarged variance in or between analytical batches and even results in a failure experiment. The successful development of the multiplex immunoassay required that the common incubation time must be suitable for all analytes. Fig. 3 showed the relationships between the fluorescence intensities and incubation times of four tumor markers. With an increasing incubation time, all the fluorescence intensities for 100 U mL^{-1} CA 125, CA 19-9, and 100 ng mL^{-1} AFP, CEA increased quickly and reached their maximum values at 25 min, indicating the maximum formation of these sandwich immunocomplexes. The optimal incubation times of our photonic suspension array for forming these sandwich immunocomplexes were much shorter than those of 1–3 h at 37 °C for the conventional microwell plate ELISA approach. Generally, incubation time depends on the kinetic characteristics of immunoreaction and mass transfer of immunoreagents. The use of photonic suspension array with consecutive shake at 37 °C can not only reduce the steric hindrance of the biomolecules and thus show faster reaction kinetic, but also produce a short diffusion distance for the immunoreagents and thus accelerated their mass transport and increased the immunoreaction rate. At the incubation time of 30 min, the fluorescence signals were stabled at the maximum values for CA 125, CA 19-9, AFP, and CEA, respectively. Since the high sensitivity of fluorescence intensities detection provided enough low detection limits for clinical application, considering the analytical time and the throughput of this assay, the incubation time of 30 min was used in the further study.

3.3. Evaluation of cross-reactivity

Cross-reactivity is a crucial analytical parameter regarding specificity and reliability of multiplex immunoassay. In our sus-

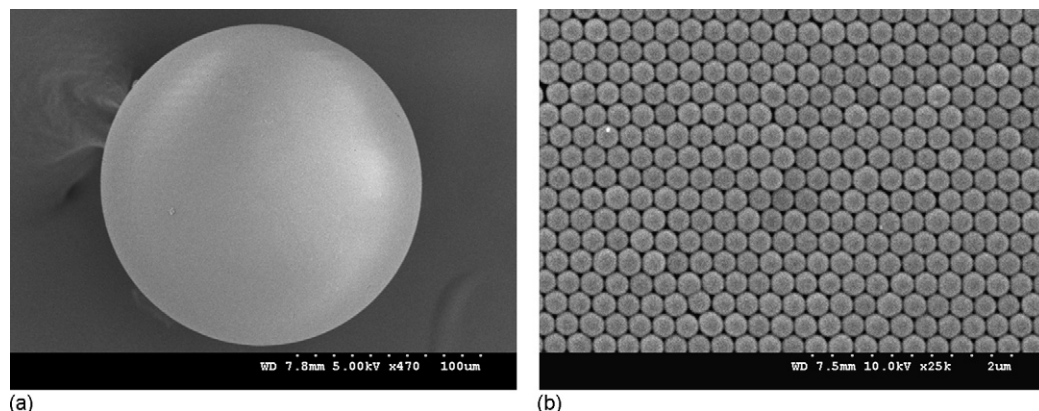


Fig. 1. SEM images of colloidal crystal beads: (a) low magnification image of a bead with diameter of 180 μm , (b) high-magnification image of the bead surface showing the hexagonal alignment of nanoparticles.

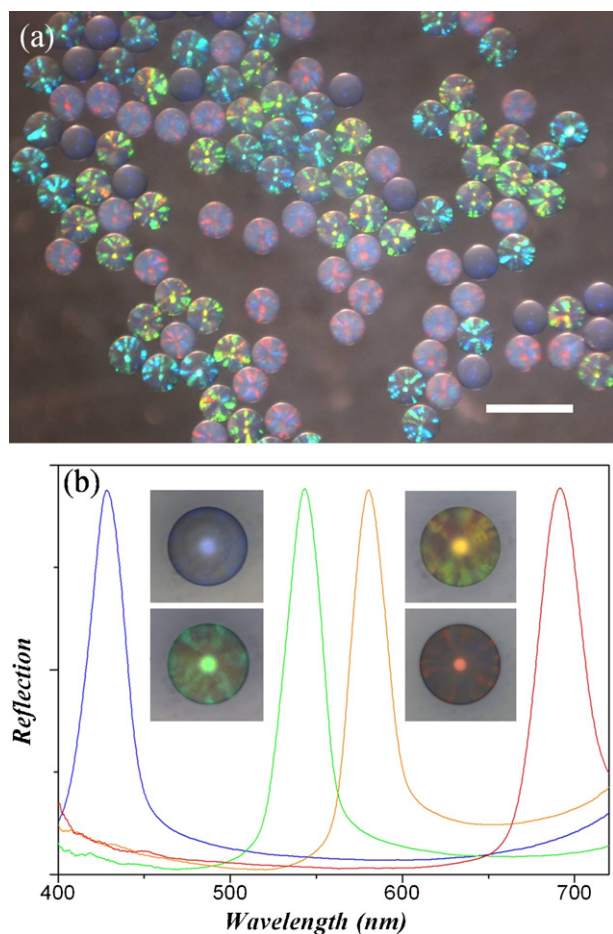


Fig. 2. (a) Three-dimensional image of four kinds of SCCBs composed of silica nanoparticles with the diameters of 195 nm, 247 nm, 264 nm and 314 nm, respectively. (b) Reflection spectra of the four kinds of SCCBs. Measurements were performed when the beads were in solution.

pension array, four tumor markers, AFP, CEA, CA125 and CA19-9, were detected in one test tube, and cross reactivity potentially occurred among the tumor markers and their noncognate antibodies was the important factor influencing the reliability of the proposed multianalyte immunosensing system. Here, the cross-

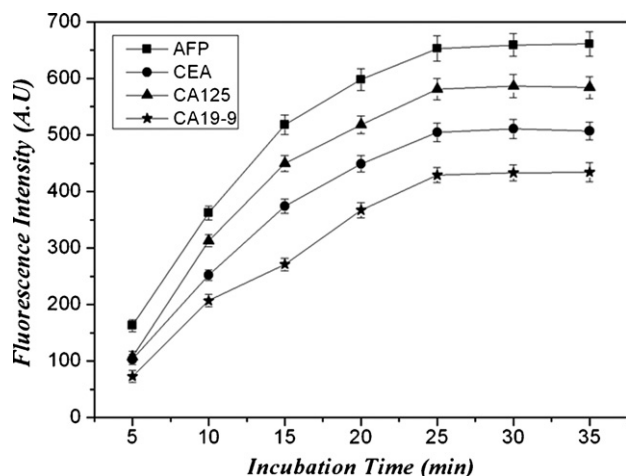


Fig. 3. Effects of incubation time on fluorescence intensities for 100 ng mL⁻¹ AFP, 100 ng mL⁻¹ CEA, 100 U mL⁻¹ CA125, 100 U mL⁻¹ CA19-9. The number of replicates at any concentration was five. Error bars represent standard deviations.

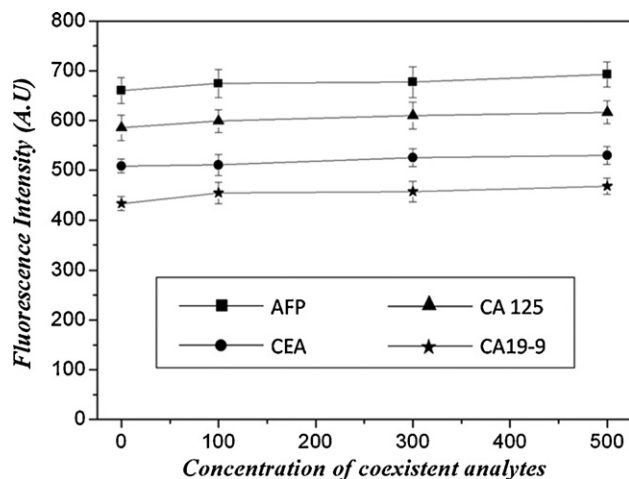


Fig. 4. The cross-reactivity among the four tumor markers and their noncognate antibodies. They were examined by comparing the fluorescence signals at a definite concentration (100 ng mL⁻¹ or U mL⁻¹) of specific analyte with increasing levels of other three coexistent analytes, respectively. The number of replicates at any concentration was five. Error bars represent standard deviations.

reactivity among the four tumor markers and their noncognate antibodies was examined by comparing the fluorescence signals at a definite concentration of specific analyte with increasing levels of other three coexistent analytes, respectively. Fig. 4 shows the effects of the coexistent analytes on the fluorescence signal for each tumor marker at a concentration of 100 ng mL⁻¹ or U mL⁻¹. Even when the concentrations of the interferents reached 500 ng mL⁻¹ or 500 U mL⁻¹ for each, the maximum changes of the fluorescence signals for 100 ng mL⁻¹ AFP, CEA and 100 U mL⁻¹ CA 125, CA 19-9 did not go beyond 4.9%, 4.4%, 5.1%, and 7.8%, respectively. The results indicated that the cross-reactivity between AFP, CEA, CA125 and CA19-9 antibodies and their noncognate antigens was negligible and the four tumor markers could be detected in one test tube without noticeable interference to others.

3.4. Analytical performance of the photonic suspension array

Under optimal conditions, we assayed routine samples of different concentrations of four tumor markers from 0 to 500 ng mL⁻¹ or U mL⁻¹. Fig. 5 shows the dose-response and calibration curves for multiplex immunoassay of AFP, CEA, CA125 and CA19-9. The curves were not linear, as are commonly observed for immunoassays, and we used curve-fitting for the calibration procedure. The limits of detection at a signal-to-noise ratio of three (3 σ) for the four tumor markers were 0.68 ng mL⁻¹, 0.95 ng mL⁻¹, 0.99 U mL⁻¹ and 2.3 U mL⁻¹, respectively. The cut-off values of the four tumor markers in clinical diagnosis are 25 ng mL⁻¹, 5 ng mL⁻¹, 35 U mL⁻¹ and 35 U mL⁻¹, respectively. Therefore, the sensitivity and detection ranges of our suspension array were enough to practical application.

To investigate the reproducibility of the newly prepared photonic suspension array, we repeatedly assayed 10 times for two different concentrations of four tumor markers, 100 ng mL⁻¹ or U mL⁻¹ and cut-off concentrations, respectively. The coefficients of variation (CVs) among 10 repetitions were 3.6% and 5.5% for 100 and 25 ng mL⁻¹ AFP; 4.6% and 7.3% for 100 and 5 ng mL⁻¹ CEA; 6.8% and 7.9% for 100 and 35 U mL⁻¹ CA125; 5.9% and 6.2% for 100 and 35 U mL⁻¹ CA19-9, respectively, which showed acceptable detection and fabrication reproducibility of the proposed immunoassay system. When the photonic suspension array was not in use, they were stored in PBS (pH 7.4) at 4 °C. No obvious change was observed

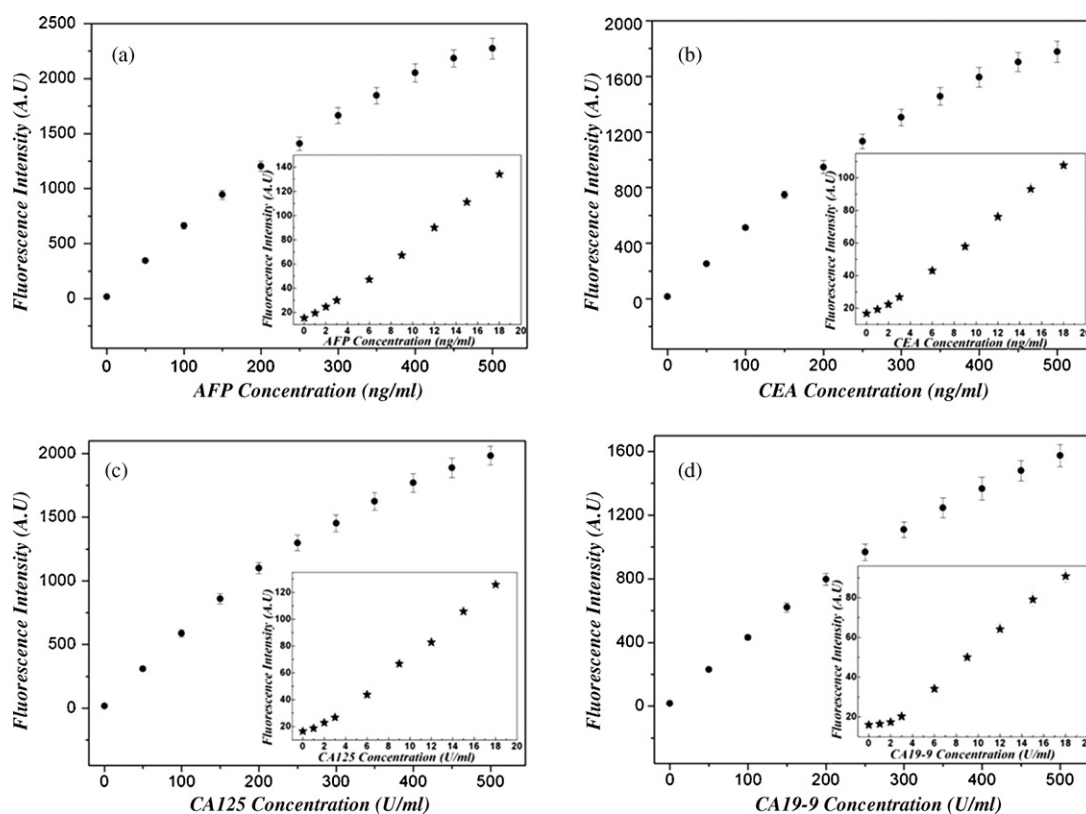


Fig. 5. Calibration plots of fluorescence intensity vs. tumor marker concentrations. Inset: low concentrations of tumor marker curves. The number of replicates at any concentration was five. Error bars represent standard deviations.

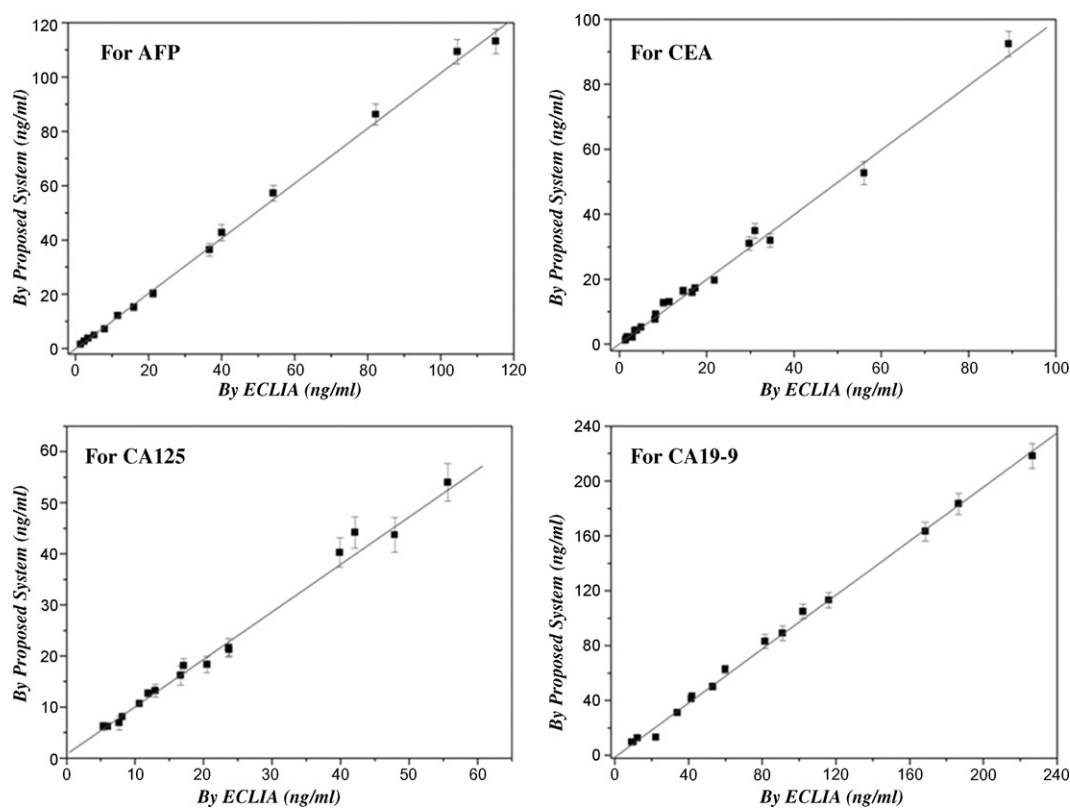


Fig. 6. Correlation between the photonic suspension array and the standard ECLIA method for the four tumor marker measurements of 26 clinical samples. The number of replicates of any clinical sample was five. Error bars represent standard deviations.

after storage for at least one year for the SCCBs without probe immobilization and at least one week for the SCCBs with probe immobilization.

3.5. Preliminary application of the photonic suspension array

To investigate the analytical reliability and application potential of the photonic suspension array for a multiplex immunoassay in clinical analysis, it was compared with the commercially proven ECLIA method; the latter was carried out with parallel single-analyte test as a reference. We examined 26 clinical serum samples. All the samples were drawn using the standard venipuncture technique followed with centrifugation from the blood cells for this purpose. The results are described in Fig. 6. The regression equations (linear) for these data are as follows (x axis, ECLIA; y axis, photonic suspension array):

$$y = -0.0578 + 1.0143 \times (r^2 = 0.9973) \text{ for AFP;}$$

$$y = 0.1487 + 0.9933 \times (r^2 = 0.9899) \text{ for CEA;}$$

$$y = 0.7522 + 0.9294 \times (r^2 = 0.9864) \text{ for CA125;}$$

$$y = -1.2566 + 0.9843 \times (r^2 = 0.9802) \text{ for CA19-9;}$$

These data showed no significant difference between the results of the two methods. It meant that our array showed good analytical reliability. In addition, the photonic suspension array consumed less analyte sample than the ECLIA, and finished the multiplex detection of the four tumor marker in one test tube, which extremely simplified the immunoassay process. However, our protocol was still time-consuming in decoding and bioassay detection, and it is still a challenge to develop the automatic device for the high throughput application of the photonic suspension array.

4. Conclusions

A novel photonic suspension array system has been designed for a multiplex detection of tumor markers. The code elements of this array were the SCCBs with a nanopatterned surface which could reduce the steric hindrance of the biomolecules and thus show faster reaction kinetic. The immunoassay results of our suspension array for AFP, CEA, CA125, and CA19-9 show acceptable accuracy, detection reproducibility, and storage stability. The sensitivity and linear ranges are also sufficient for practical application. Therefore, the photonic suspension array is especially suitable for detection

of disease marker for diagnosis and screening purpose. Adaptation of this methodology to other important multianalyte immunoassay systems for the areas of human health, environmental testing, drug monitoring and toxin detection is currently in progress.

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