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# Simultaneous fluoroimmunoassay of two tumor markers based on CdTe quantum dots and gold nanoclusters coated-silica nanospheres as labels

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In this study, a novel fluoroimmunoassay protocol for simultaneous detection of two tumor markers is described. The new approach employed magnetic bead as carrier for the antibody immobilization, while CdTe quantum dots (CdTe QDs) and gold nanoclusters (Au NCs) coated-silica nanospheres were used as labels. Carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP) were adopted as model proteins. After a typical sandwich-type immunoreaction, the immunocomplex exhibited two distinguishable fluorescence peaks at 550 nm and 655 nm corresponding to CdTe QDs and Au NCs, respectively. Under optimal conditions, fluorescence intensities were linearly increased to the concentration of CEA and AFP in range of 0.1-400 ng $mL^{-1}$, the detection limit of fluoroimmunoassay is 0.04 ng $mL^{-1}$ for CEA and 0.08 ng $mL^{-1}$ for AFP, respectively. The proposed method was evaluated with human serum, and the determination values obtained were accordance with reference methods reported. These results demonstrated that the new method can be applied to the determination of two tumor markers in clinical samples.

## 1 Introduction

Recently, multiplexed immunoassay which permits simultaneous detection of multiple biomarkers in single run has caused considerable attention[1-3] because the detection results from single tumor marker give lower diagnosis value. To date, various methods for multiplexed biomarkers analasis have been reported including electrochemical and optical methods.[4-9] Among these methods, fluorescence immunoassay has been recognized as a suitable strategy due to its high sensitivity and stable properties. Thus, it is very necessary to develop a rapid, sensitive and selective fluoroimmunoassay for multiplexed biomarkers detection.

To construct high sensitive multiplexed fluoroimmunoassay, the first challenge is to search fluorescent tags whose signals are distinguishable. Organic dyes and lanthanide complexes are often used as fluorescent tags in fluoroimmunoassay. However, organic dyes are suffered from the low quantum yields, poor photochemical stability, photobleaching and are limited in multiplexed fluoroimmunoassay;[10, 11] Lanthanide complexes can exhibit distinguishable narrow emissive signal at different wavelengths,[12, 13] which make them suitable as labels for the multiple biomarkers determination. For example, Wu's group[14] used the chelates of europium ($Eu^{3+}$) and samarium ($Sm^{3+}$) as fluorescent labels and simultaneous detected a-fetoprotein (AFP) and the free b-subunit of human chorionic gonadotropin (free β-hCG) in human serum. The detection limit obtained was 0.05 ng $mL^{-1}$ for AFP and 0.08 ng $mL^{-1}$ for free β-hCG. Unfortunately, lanthanide chelates are limited in thermal stability and mechanical stability.

Quantum dots (QDs) are semiconductor nanocrystals in size range of 1-10 nm. Although physically larger than organic dyes and fluorescent proteins, they own some unique properties including high fluorescence efficiency, superior photostability, size dependent emission wavelength turnability. These advantages make QDs potential candidates as signal tags in immunoassay field[15-20]. Moreover, when QDs are functionalized with bio-recognition molecules such as antibodies, [21]peptides[22] and oligonucleotides,[23] it can also emit strong fluorescence. Based on the feature, QDs will become attractive fluorescent probes in biolabeling and imaging filed. Noble metal nanoclusters (NCs), which possess intense fluorescence and good biocompatibility, have gained a tremendous interest in bioanalytical filed during last decade.[24, 25] Especially, NCs with red emission exhibit some novel advantages such as maximal penetration in tissue ability and high signal-to-noise ratio. Hence, NCs with red emission are considered as ideal fluorescent probes for biolabelling and bioimaging.[26-28] Previous studies showed QDs or NCs were easily agglutination and

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lead to fluorescence intensity decrease. Thus, a key issue using QDs or NCs as fluorescent labels is how to avoid QDs or NCs agglutination. We've learned from literatures, an effective way to solve the problem is to deposit QDs or NCs on the surface of silica nanoparticles or to embed them into hollow silica nanospheres (Si).[29] The QDs or NCs not only exhibit good dispersion,[30-33] but also own excellent biocompatibility. To our knowledge, the fluoroimmunoassay for simultaneous detection of multiple tumor markers has not been reported based on CdTe quantum dots and gold nanoclusters coated- silica nanospheres (Si/CdTe QDs and Si/Au NCs) as labels.

In present work, a novel sandwich-type fluoroimmunoassay protocol for the simultaneous measurement of two tumor markers is described. Magnetic bead (MB) was used as carrier for two antibodies immobilization (in this work, it serves as a high throughput platform for detection as well as a rapid and efficient separation means). Si/CdTe QDs and Si/Au NCs were as labels for carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP) detection. After sandwich-type immunoreactions, the immunocomplex obtained exhibits two distinguish fluorescence peaks at 550 nm (corresponding to CdTe QDs) and 655 nm (corresponding to Au NCs) in fluorescence spectrum, respectively. Fluorescence intensities are linearly related to the concentration of CEA and AFP in range of 0.1-400 ng $mL^{-1}$. The proposed method was evaluated with human serum. The detection results from the proposed method were accordance with the reference methods reported, indicating the fluoroimmunoassay method can be applied to simultaneous detection of tumor biomarkers in clinical samples.

## 2 Experimental

### 2.1 Reagents and instrumentation

The Carcinoembryonic antigen (CEA), anti-CEA antibody ($Ab_{CEA}$), α-fetoprotein (AFP) and anti-AFP antibody ($Ab_{AFP}$) were purchased from Biocell Biotech. Co., Ltd (Zhengzhou, China). Carboxyl-functionalized magnetic bead (MB) was purchased from Huier Nanotech. Co., Ltd (Luoyang, China). Bovine serum albumin (BSA), Tween-20, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 3-Aminopropyltriethoxysilane (APTES), Tetraethoxysilane (TEOS) were purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). All the other reagents were of analytical grade and were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Phosphate buffered solution (PBS) was prepared by mixing $NaH_2PO_4$ and $Na_2HPO_4$. Blocking solution was 1% BSA. Twice-distilled water was used for solution preparation. The clinical serum samples were from the clinical laboratory of the Yiji Shan Hospital (Wuhu, China). All experiments were performed in compliance with the relevant laws and institutional guidelines of the Ethics Committee of the Hospital and informed consent was obtained from the patients who provided the human samples.

The morphologies of various nanomatrials and nanocomposites were obtained at a transmission electron microscope (TEM, Hitachi-800, Japan). All fluorescence measurements were carried out at an F-4500 fluorescence spectrophotometer (Hitachi, Japan). Fluorescence imaging was performed on a confocal laser scanning Leica TCS SP8 microscope with a pulsed near infrared laser for multiphoton excitation. Laser wavelength is 405 nm for Si/CdTe QDs and 488 nm for Si/Au NCs. The corresponding laser power is 1mW*10% and 5mW*10%, respectively. The collection window is 533 nm-575 nm and 632 nm-675 nm, respectively.

### 2.2 Preparation of magnetic bead-antibody bioconjugates

Two magnetic bead-antibody bioconjugates ($MB/Ab_{CEA}$ and $MB/Ab_{AFP}$) were prepared according to the method described by Zhao et al [34] with a slight modification. Here, we described the preparation process of $MB/Ab_{CEA}$. Briefly, 5 mg MB was suspended in 1.2 mL pH 7.4 PBS solution containing 0.05% Tween-20 under ultrasonication, Subsequently, 200 μL of 400 μg $mL^{-1}$ $Ab_{CEA}$,100 μL of 10 mg $mL^{-1}$ EDC and 200 μL of 10 mg $mL^{-1}$ NHS were added into suspension above in sequence. After 3 h, the reaction product was magnetic separated, the supernatant was decanted. Thus, excess antibody, EDC and NHS were removed from the suspension. The precipitates collected were re-suspended in 1.0 mL pH 7.4 PBS containing 1% BSA and stored at 4 ℃ for further used. The product was denoted as $MB/Ab_{CEA}$.

Similarly, the $MB/Ab_{AFP}$ was prepared according to the similar process of $MB/Ab_{CEA}$ described above.

### 2.3 Preparation of two labels

To prepare two labels, silica nanospheres (Si) were first prepared according to the literature.[35] and its diameter was estimated about 200±3.0 nm using TEM technology (Scheme.1A). The synthesis of water-soluble CdTe QDs and BSA-protected Au NCs were carried out according to procedure described previously.[36, 32] The detailed procedure of CdTe QDs and Au NCs were shown in supporting information. Scheme.1A illustrated the protocols of Si/CdTe QDs /$Ab_{CEA}$ or Si/Au NCs/$Ab_{AFP}$ labels.

#### 2.3.1 Preparation of Si/ CdTe QDs and Si/Au NCs

For the preparation of Si/CdTe QDs and Si/Au NCs, silica nanospheres were first functionalized. Briefly, 0.02 g of silica nanospheres were first dispersed in 2 mL ethanol under ultrasonication. Next, 0.4 mL APTES was added. After 6 h, the suspension was centrifuged and rinsed with ethanol repeatedly for three times. Thus, the precipitates collected were amino-functionalized silica nanospheres. The functionalized silica nanospheres were re-dispersed in a mixture containing 2.0 mL CdTe QDs (or Au NCs) and 200 μL of 10 mg $mL^{-1}$ EDC. After 12 h, unbound CdTe QDs (or Au NCs) were removed by successive centrifugation, and the precipitates were rinsed several times with distilled water. Finally, the products obtained were re-dispersed in water until a final volume of 1.0 mL.

#### 2.3.2 Labeling of antibody

In this study, two different antibodies were labeled on Si/CdTe QDs or Si/Au NCs, respectively. Here, we described the labeling of $Ab_{CEA}$ on Si/CdTe QDs in detail. 200 μL of 20 μg $mL^{-1}$ $Ab_{CEA}$, 100 μL

of 10 mg $mL^{-1}$ EDC and 100 µL of 10 mg $mL^{-1}$ NHS were injected into 1.0 mL of the Si/CdTe QDs suspension in sequence. After 2 h, the mixture was treated with high speed centrifugation and the supernatant were decanted. Precipitate was rinsed with pH 7.4 PBS and centrifuged several times. After that, the product was blocked with 2.0 mL of 1% BSA solution. Then the suspension was high speed centrifuged and washed, the resultant Si/CdTe QDs/$Ab_{CEA}$ bioconjugate was re-dispersed in 2.0 mL pH 7.4 PBS and stored at 4 ℃ for later use.

Similarly, the bioconjugate of Si/Au NCs/$Ab_{AFP}$ was obtained according to the procedure described above. During the process, Si/CdTe QDs suspension was replaced with Si/Au NCs suspension.

#### 2.4 Fluoroimmunoassay protocol

Scheme.1B represents the principal of fluorescence immunoassay based on Si/CdTe QDs and Si/Au NCs as signal tags. Initially, 50 µL of CEA and AFP with different concentrations were added to test tubes. Then, 25 µL of MB/$Ab_{CEA}$, 30 µL of MB/$Ab_{AFP}$, 200 µL of Si/CdTe QDs/$Ab_{CEA}$ and 200 µL of Si/Au NCs/$Ab_{AFP}$ were added sequentially. After incubation for 40 min at room temperature, the immunocomplexes were separated from solution under a samarium–cobalt magnet, followed by addition of 200 µL of pH 7.4 PBS. Finally, the fluorescence signals were obtained at the excitation wavelength of 380 nm on an F-4500 fluorescence spectrophotometer.

**Scheme.1** (A) Preparation process of labels of Si/CdTe QDs and Si/Au NCs. (B) Fluoroimmunoassay protocol of the simultaneous detection of CEA and AFP.

## 3 Results and discussion

#### 3.1 Characterization of Si/CdTe QDs and Si/Au NCs

In this study, CdTe QDs or Au NCs were immobilized on the surface of silica nanospheres via amide reaction. Simply, APTES was first coupled to the hydroxyl group on silica nanospheres to yield an amino-terminated monolayer. Subsequently, the carboxylic groups located on the surface of CdTe QDs or Au NCs reacted with amino groups on the surface of silica nanospheres in the presence of EDC and NHS. Thus, CdTe QDs or Au NCs was assembled on the surface of silica nanospheres. TEM and fluorescence spectrum were employed to investigate the Si/CdTe QDs or Si/Au NCs, and the results were shown in Fig.1. Two distinguish ufluorescence signal at 550 nm and 655 nm could be observed after CdTe QDs and Au NCs was assembled on the surface of silica nanospheres, which are consistent with peak position of CdTe QDs or Au NCs. Furthermore, TEM images exhibited numerous, individual, dark “islands”, indicating the CdTe QDs or Au NCs was distributed homogeneously on the surface of silicon nanospheres.

**Fig.1** (A, B) The fluorescence emission spectra of Si/CdTe QDs and Si/Au NCs (excitation wavelength, 380nm); the inset photos show the corresponding fluorescence colors under a 365 nm UV lamp, respectively. (C, D) TEM images of Si/CdTe QDs and Si/Au NCs, respectively.

#### 3.2 Characterization of Si/CdTe QDs /$Ab_{CEA}$ and Si/Au NCs/$Ab_{AFP}$

Fluorescence spectrum and fluorescence microscopy technology were used to characterize the Si/CdTe QDs/$Ab_{CEA}$ and Si/Au NCs/$Ab_{AFP}$ bioconjugates. And these results were shown in Fig.2A and 2B. In presence of antibody, fluorescence intensity decreased a little in contrast to Si/CdTe QDs (5% decreased in signal) and Si/Au NCs (8% decreased in signal). This reason may be the antibodies on the surface of CdTe QDs or Au NCs shielded the fluorescence signal.

In order to confirm the fluorescence signals were from Si/CdTe QDs/$Ab_{CEA}$ or Si/Au NCs/$Ab_{AFP}$. We designed three different experiments. First, Si/CdTe QDs/$Ab_{CEA}$ (or Si/Au NCs/$Ab_{AFP}$) was incubated with 20 ng $mL^{-1}$ CEA (or AFP) to form Si/CdTe QDs/$Ab_{CEA}$-$Ag_{CEA}$ bioconjugates (or Si/Au NCs/$Ab_{AFP}$-$Ag_{AFP}$ bioconjugates) ; Second, the MB/$Ab_{CEA}$ (or MB/$Ab_{AFP}$) was incubated with 20 ng $mL^{-1}$ CEA (or AFP), followed by soaking in Si/CdTe QDs (or Si/Au NCs) suspension to produce MB/$Ab_{CEA}$-$Ag_{CEA}$ (or MB/$Ab_{AFP}$-$Ag_{AFP}$); Third, the MB/$Ab_{CEA}$ (or MB/$Ab_{AFP}$) was incubated with 20 ng $mL^{-1}$ CEA (or AFP), followed by soaking in Si/CdTe QDs/$Ab_{CEA}$ (or Si/Au NCs/$Ab_{AFP}$) suspension to produce Si/CdTe QDs/$Ab_{CEA}$-$Ag_{CEA}$/MB/$Ab_{CEA}$ composites (or Si/Au NCs/$Ab_{AFP}$-$Ag_{AFP}$/MB/$Ab_{AFP}$ composites). After centrifugation or magnetic separation, the precipitates of three different conditions were washed and dispersed in 0.5 mL pH7.4 PBS. 2 µL suspensions above were dropped on the microscope slides and were visualized respectively. The results obtained were shown in Fig. 2C- Fig. 2H. It could be seen that green and red fluorescence were observed in presence of first and third conditions, respectively. In contrast, no fluorescence was observed in presence of second condition because Si/CdTe QDs (or Si/Au NCs) were washed off during magnetic separation process. These facts showed fluorescence signals were from Si/CdTe QDs/$Ab_{CEA}$ or Si/Au NCs/$Ab_{AFP}$ bioconjugates.

**Fig.2** Fluorescence emission spectra of Si/CdTe QDs/$Ab_{CEA}$ (A) and Si/Au NCs/$Ab_{AFP}$ (B). Excitation wavelength: 380 nm. Fluorescence microscopy images in various condition: (C) Si/CdTe QDs/$Ab_{CEA}$-$Ag_{CEA}$, (D) the MB/$Ab_{CEA}$-$Ag_{CEA}$ incubated with Si/CdTe QDs, (E) MB/$Ab_{CEA}$-$Ag_{CEA}$ incubated with Si/CdTe QDs/$Ab_{CEA}$, (F) Si/Au NCs/$Ab_{AFP}$-$Ag_{AFP}$, (G) the MB/$Ab_{AFP}$-$Ag_{AFP}$ incubated with Si/Au NCs, (H) MB/$Ab_{AFP}$-$Ag_{AFP}$ incubated with Si/Au NCs/$Ab_{AFP}$.

#### 3.3 Optimization of experimental conditions

In order to obtain good analytical performance, some experiment conditions were optimized including incubation time and concentration of magnetic beads. Fig. 3A showed the relationship between fluorescence intensity and incubation time. It could be observed clearly that the fluorescence intensity reached maximum when incubation time was 40 min, and it did not noticeably increase over 40 min. Therefore, 40 min incubation was selected in this study.

The concentration of MB/Ab bioconjugate was investigated

and the results were shown in Fig. 3B. It could be observed that the fluorescence intensities corresponding to CEA and AFP were all increased as the amount of $MB/Ab_{CEA}$ and $MB/Ab_{AFP}$ increased from 0-250 μg $mL^{-1}$ and 0-300 μg $mL^{-1}$, respectively. After that, they kept a stable value. The reason may be that the antibody immobilized on the surface of MB captured the maximum amount of analytes. Hence, 250 μg $mL^{-1}$ $MB/Ab_{CEA}$ and 300 μg $mL^{-1}$ $MB/Ab_{AFP}$ were chosen in this study.

**Fig.3** Effect of the incubation time (A), and the concentration of magnetic beads (B) on fluorescence intensity. $C_{CEA}$= $C_{AFP}$= 50 ng $mL^{-1}$.

### 3.4 Analytical performance

Under the selected conditions, the immunoassay method was employed to detect a series of CEA and AFP standards with different concentrations (0.1, 10, 20, 100, 200, and 400 ng $mL^{-1}$). The results obtained were shown in Fig. 4. It could be observed that the fluorescence intensities were increased gradually with increasing concentration of CEA and AFP, and two good calibration curves were obtained in the range of 0.1 to 400 ng $mL^{-1}$. For CEA, the linear regression equation was $I_F$=132.51 + 9.39C (ng $mL^{-1}$) with a linear regression coefficient of 0.9971. For AFP, the linear regression equation was $I_F$=79.23+ 2.63C (ng $mL^{-1}$) with a linear egression coefficient of 0.9967. The detection limit of 0.04 ng $mL^{-1}$ for CEA and 0.08 ng $mL^{-1}$ for AFP was obtained (at S/N=3). The high sensitivity may be ascribed to the large surface area of magnetic beads and silica nanospheres for loading amount of antibody, which would enhance the chances of interaction between the antigen and antibody.

**Fig.4** Fluorescence spectra of the immunoassay in presence of different concentrations of CEA and AFP. From the bottom to the top: 0.1, 10, 20, 100, 200, 400 ng $mL^{-1}$.

### 3.5 Analytical application in human serum

To investigate the applicability of the developed fluoroimmunoassay method in practical analyses, the recovery experiments were performed by spiking different amounts of CEA and AFP with known concentrations to the healthy human serum. The standard addition method was employed. The recoveries obtained were within 94.5-104.2% and 95.2%-103.1%, respectively. The results indicated that the immunoassay method is suitable for serum sample (seen in Table 1).

In order to further investigate the feasibility of the developed immunoassay in clinical applications, the blood samples from venous blood were tested using the fluorescence immunoassay. The obtained values were compared with that of ELISA detection (Seen in Table 2), indicating the fluoroimmunoassay can be applied to serum sample analysis.

**Table 1** Recovery results of CEA and AFP in human serum samples.

**Table 2** Comparison of the determination results of CEA and AFP in human serum using the proposed and reference methods.

## Conclusions

In this work, we developed a high sensitive fluoroimmunoassay for the simultaneous detection of CEA and AFP in human serum. Magnetic beads were used as carrier for antibody immobilization and Si/CdTe QDs and Si/Au NCs bioconjugates were employed as signal labels. The large surface area of magnetic beads and silica nanospheres enhanced the amount of antibody immobilization, as well as increased the signal intensity. As a result, the fluoroimmunoassay possessed high sensitivity and good selectivity.

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**Scheme.1**

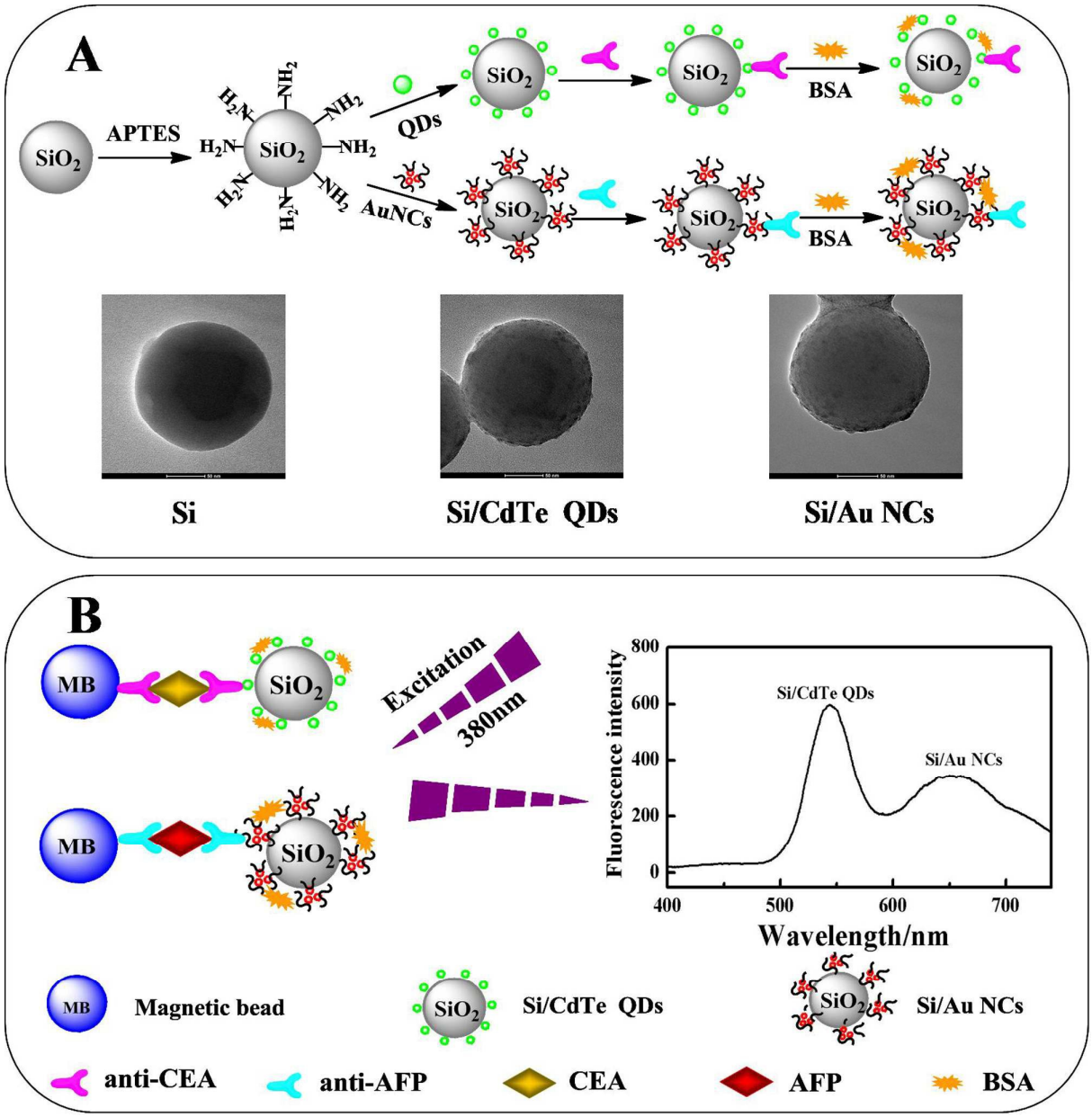


**Fig.1**

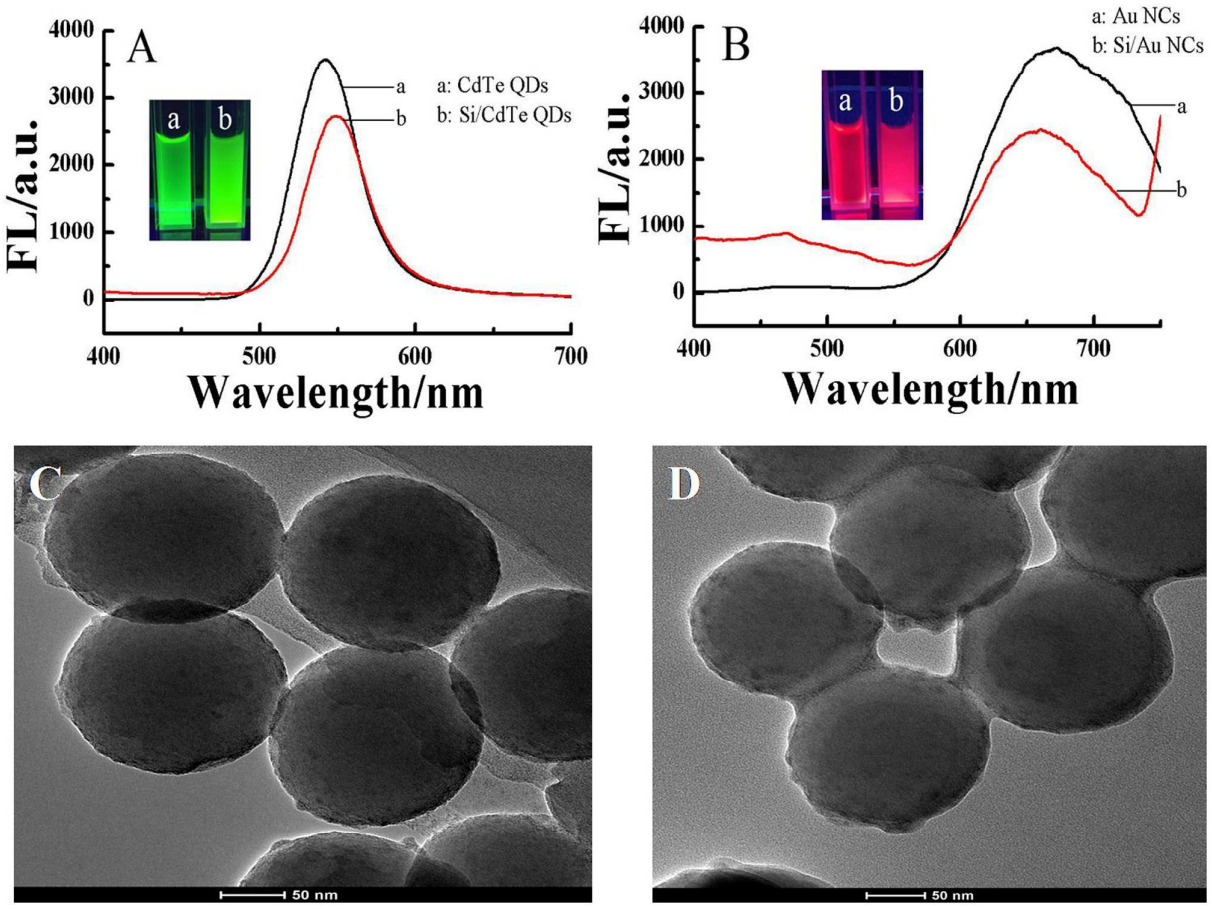


**Fig.2**

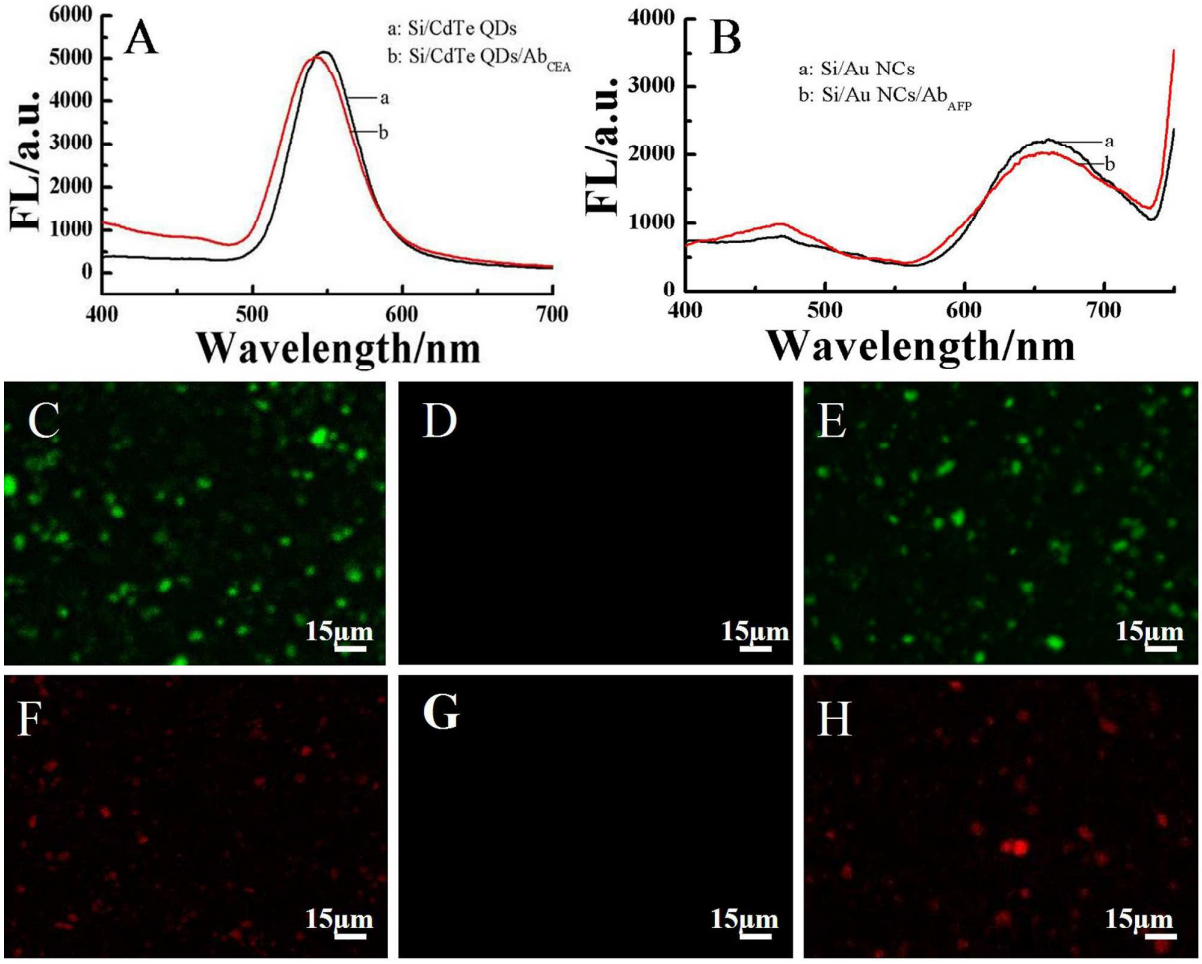


**Fig.3**

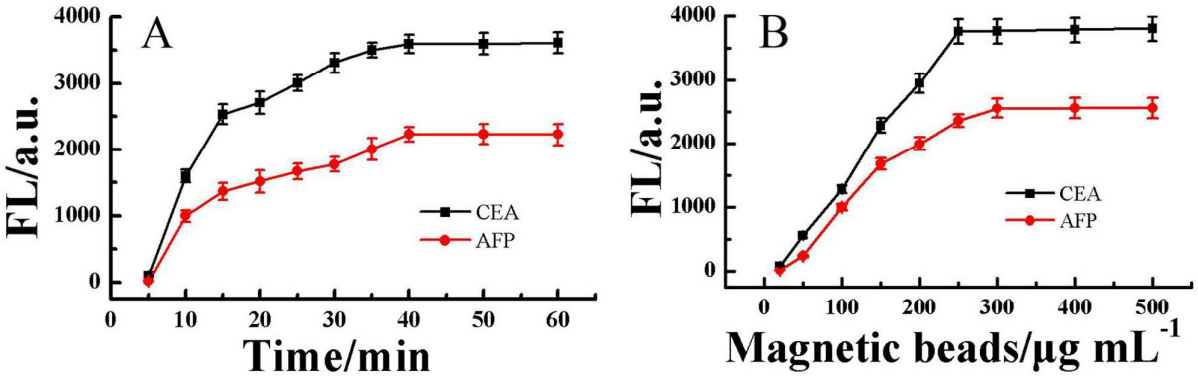


**Fig.4**

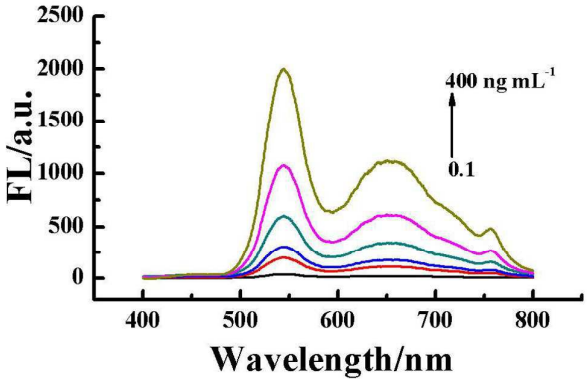


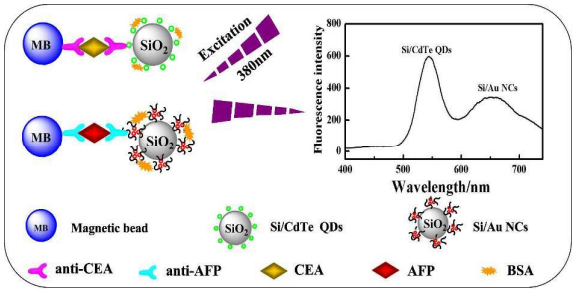
**Table 1**

| Serum samples | add (ng mL$^{-1}$) | | found (ng mL$^{-1}$) | | Recoveries (%) | |
|---|---|---|---|---|---|---|
| | CEA | AFP | CEA | AFP | CEA | AFP |
| 1 | 10.00 | 10.00 | 9.45 | 9.86 | 94.5 | 98.6 |
| 2 | 20.00 | 20.00 | 20.26 | 19.03 | 101.3 | 95.2 |
| 3 | 40.00 | 40.00 | 38.68 | 41.24 | 96.7 | 103.1 |
| 4 | 80.00 | 80.00 | 79.04 | 78.31 | 98.8 | 97.9 |
| 5 | 160.00 | 160.00 | 166.76 | 163.56 | 104.2 | 102.2 |

**Table 2**

| Serum no | Multiplexed immunoassay[a] (ng mL$^{-1}$) | | ELISA (ng mL$^{-1}$) | | Relative deviation (%) | |
|---|---|---|---|---|---|---|
| | CEA | AFP | CEA | AFP | CEA | AFP |
| 1 | 10.3±0.35 | 10.5±1.02 | 10.0 | 10.0 | +3.0 | +5.0 |
| 2 | 24.4±0.68 | 25.5±0.98 | 25.0 | 25.0 | - 2.4 | +2.0 |
| 3 | 50.8±1.59 | 51.3±2.26 | 50.0 | 50.0 | +1.6 | +2.6 |
| 4 | 103.8±3.71 | 98.5±2.18 | 100.0 | 100.0 | +3.8 | - 1.5 |
| 5 | 196.4±4.26 | 195.6±3.14 | 200.0 | 200.0 | - 1.8 | - 2.2 |

## Graphic Absract




Fluoroimmunoassay for simultaneous detection of CEA and AFP