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PAPER

A fluorescence-based colorimetric droplet platform for biosensor application to the detection of α-fetoprotein†

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Droplet methods have been successfully applied in DNA hybridization analysis and protein-protein interaction. Existing assay methods implemented in droplet platforms are severely limited by expensive and high-maintenance equipment. As a convenient detection method, colorimetry provides a new path for microscale assay since it can enhance assay efficiency and simplify the detection procedure. Here, a microscale immunoassay for α-fetoprotein (AFP) was developed for the first time by the incorporation of colorimetry and droplet platform. $Ru(bpy)_2(mcbpy\text{-}O\text{-}Su\text{-}ester)(PF_6)_2$ complex (Ru) was coupled with the monoclonal antibody (Ab) of AFP to form a stable red Ru–Ab complex both as a quencher for green CdTe quantum dots (QDs) and as a capture probe for AFP. In the absence of AFP, the mixed droplet showed a red color. With the increase of AFP concentration, the color change of the droplet was from red to green as a result of the competition of AFP with QDs for Ru–Ab. The biosensor exhibited not only good sensitivity and specificity for AFP with a detection limit of 0.06 ng ml^{-1}, but also satisfactory performance in diluted human sera with a detection limit of 0.4 ng ml^{-1}. Notably, a visual droplet platform for screening cancer biomarkers by the naked eye based on this principle is anticipated.

1. Introduction

Ultrasensitive detection of cancer biomarkers has received great attention due to its importance for early disease diagnostics.[1–3] As a well-known tumor marker, α-fetoprotein (AFP) is a serum biomarker of hepatocellular carcinoma, interest in AFP analysis has been increased in recent years.[4–11] Although existing methods for protein detection show good sensitivity and specificity, most of them rely on a static solid/liquid interface reaction, leading to long incubation times, repetition of the washing process and involvement of protein immobilization and blocking proteins. Moreover, protein immobilization can weaken the biological activities of the protein and interfere significantly with protein-protein interactions.[12,13] DeMello's group proposed using a droplet system to study protein-protein interactions based on fluorescence resonance energy transfer (FRET).[12] On one hand, this work demonstrated that the whole process is free of immobilization, incubation and washing time in a high-throughput manner. On the other hand, a droplet, as an isolated reactor can not only accelerate reagents mixing and shortening the reaction time, but also eliminates the dispersion and cross-contamination of reagents to a greater extent, as well as act to control the solution volume precisely. This suggests that the droplet has the ability to act as an excellent platform when applied in a biosensor for protein detection with the advantage of low consumption of reagents.

However, the work of DeMello is not suitable for large proteins because of the distance limitation required by FRET. In addition, since the droplet moves at high speed, the experimental process requires expensive instruments, which usually limit droplet usage in protein assays. In the first problem, a dual fluorescence model for virus detection in tubes was presented ,[14] the process was unrestricted by protein size. In the second problem, with the development of manipulation technologies,[15–17] the droplet can be transported, mixed, trapped and released on demand. Therefore, protein detection can be achieved by a common imaging device. On the basis of these advantages, a more universal and simpler droplet platform can be built for the analysis of protein-protein interaction. Further, this droplet platform can be applied in the protein biosensor as an alternative to the traditional detection mode in tubes. However, the droplet technique has not yet been reported as a new quantitative tool in protein biosensors.

The dual color change observed has high resolution to the naked eye compared to the color change in homochromatism, and it is difficult for conventional colorimetry to be performed in a droplet with satisfactory results. To construct a sensitive droplet sensor for AFP, a colorimetric detection model

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combined with fluorescence techniques as the signal output is promising.[18] Wang's group proposed a fluorescence-based colorimetric detection model for oxygen detection by choosing quantum dots (QDs) as a reference color for another dye.[19] However, it was not the best choice to take the CdTe QDs as a reference color in the droplet sensor because the fluorescence intensity of most of dyes is much lower than that of QDs at the same concentration. If general dyes are used as the colorimetric method it will reduce the sensitivity of protein detection in the droplet. It is necessary, therefore, to find a stable fluorescent material, which can substitute QDs to act as a reference color. Ruthenium complexes can provide very stable fluorescence with an emission peak around 610 nm.[20] Besides, excitation with QDs at the same wavelength presents different colors so providing a good pair of color change. Additionally, some ruthenium complexes can be coupled with a protein or directly interact with double-stranded DNA and thus serve as a quencher for QDs simultaneously, which is favorable to simplify the experimental process.[21] Inspired by this, a fluorescence-based colorimetric droplet platform applied in a protein biosensor was realized by adopting $(Ru(bpy)_2(mcbpy\text{-}O\text{-}Su\text{-}ester)(PF_6)_2$ (Ru) ruthenium complex to act as quencher and reference color for QDs.

We herein report a fluorescence-based colorimetric immunoassay for AFP determination *via* conjugating the monoclonal antibody of AFP with Ru to form a Ru–Ab complex. The design of chip and sensor strategy is depicted in Fig. 1. The Ru–Ab serves as both a capture probe for AFP antigen and as a quencher for QDs. To establish an excellent colorimetric droplet sensor for protein detection, green CdTe QDs are used in our work (Fig. S1ESI†). With the help of a microvalves technique, QDs, Ru–Ab and AFP droplets are generated, fused, trapped and photographed on demand. A series of AFP droplets with stepwise concentration gradient are created on a single chip. Moreover, the sensor platform is directly applied to quantitative AFP determination of clinical samples by visualization. The sensor can be used as a universal colorimetric platform for any protein by coupling Ru to the corresponding antibody. Based on this sensor strategy, multiplexed biomarkers assays can be achieved in droplet by increasing the channels of the chip.

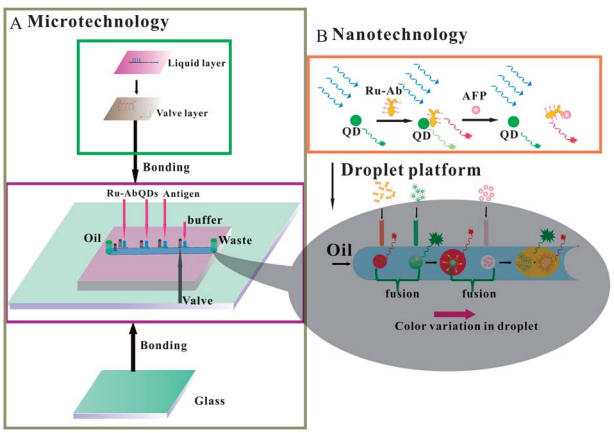

Fig. 1 (A) Schematic picture of the microfluidic droplet platform and (B) schematic illustration of a microscale colorimetric immunoassay for AFP based on QDs and Ru–Ab fluorescent probes and microfluidic droplet biosensor.

2. Experimental

2.1 Apparatus and reagents

Fluorescence spectra data are collected with a RF-5301PC fluorescence spectrophotometer (Shimadzu, Tokyo, Japan).

Water-soluble *N*-acetylcysteine (NAC)-capped CdTe quantum dots (QDs) were synthesized in accordance with the procedure in ref. 22. Mineral oil, Span 80, bis (2,2′-bispyridine)-4′-methyl-4-carboxy bipyridine-ruthenium *N*-succinimidyl ester-bis (hexafluorophosphate) ($C_{36}H_{29}F_{12}N_7O_4P_2Ru$) were purchased from Sigma-Aldrich. Ru–Ab conjugation was prepared according to ref. 14. The α-fetoprotein (AFP) and mouse monoclonal antibody to AFP were supplied by Jingtian Biotech Co. Ltd (Shanghai, China). Human sera were provided by the School of Medicine, Wuhan University and were not handled further. Some serum samples were obtained from healthy individuals and the others were obtained from cancer patients. All amino acids, human serum albumin (HSA), immunoglobulin G and transferrin were supplied by Newprobe Biotechnology Co. Ltd (Beijing, China). Sodium phosphate dibasic anhydrous (Na_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), calcium chloride anhydrous ($CaCl_2$), magnesium nitrate hexahydrate ($Mg(NO_3)_2 \cdot 6H_2O$) were supplied by Sinopharm Chemical Reagent (Shanghai, China). The solutions of Ru–Ab, QDs, and AFP were prepared in 15 mM PBS buffer (pH 8.0, 10 mM NaCl). All aqueous solutions were prepared using ultrapure water (Mill-Q, Millipore, 18.2 MΩ resistivity). All fluorescence measurements were made with a spectrometer (Shimadzu, Japan). All droplet images were acquired by an inverted fluorescence microscope (Axio Observer.A1, Zeiss, Germany) with a 10× objective and a Spot RT3 charge-coupled device (CCD, Diagnostic Instruments, Inc, USA).

2.2 Procedure for AFP detection in a droplet

A three-layer PDMS microfluidic chip was designed by soft-photolithography. The details of chip fabrication, generation, control and fusion of droplets in this microfluidic device are provided in ref. 23. Ru–Ab and QDs droplets were generated and fused as a whole, and then the droplet of AFP was mixed into the former droplet. Finally, the well-mixed droplet was immobilized at the detection region and imaged. The fluorescence intensity of droplet was acquired by Image-Pro Plus software.

3. Results and discussion

3.1 The principle of colorimetric sensing in a droplet

The scheme for this AFP droplet sensor is illustrated in Fig. 1B. For the colorimetric droplet sensor, by pneumatic microvalves technique in the absence of AFP, only the Ru–Ab and QDs droplet are fused together. According to the zeta potential shown in Fig. S2,† the electrostatic interaction makes the positively charged Ru–Ab (+1.09 mV) complex close to the surface of the negatively charged QDs (−11.2 mV), and the emission character of QDs is efficiently suppressed. As a result, a single fluorescence of the Ru–Ab complex appears, and the droplet displays the color of Ru. In the presence of AFP, Ru–Ab and AFP first form the negatively charged complex Ru–Ab-AFP

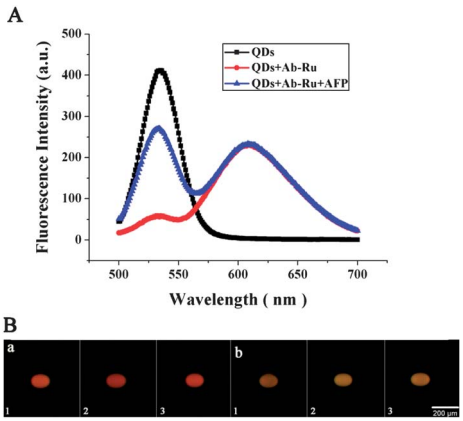

Fig. 2 (A) Fluorescence emission spectra of QDs (22 nM), QDs-Ru–Ab (15 μg ml^{-1}) and QDs-Ru–Ab-AFP (25 ng ml^{-1}) in 15 mM PBS solution (pH 8.0; $\lambda_{ex} = 460$ nm). (B) Color responses of quenched droplets in the absence (a) and presence of AFP with the concentration of 10 ng ml^{-1} (b). The concentrations of QDs and Ru–Ab were 3.5×10^{-6} M and 0.3 mg ml^{-1}, respectively. The VRs of QDs/Ru–Ab/AFP were 1 : 3 : 3. The average size of a droplet was 0.2 nl.

(−9.08 mV, Fig. S2†) due to the strong and specific affinity of AFP to Ru–Ab, which is far greater than the electrostatic interactions of Ru–Ab and QDs. As a result, the repulsive interactions between Ru–Ab-AFP and QDs position Ru–Ab far away from the QDs. Meanwhile, the luminescence of QDs is restored and a dual fluorescence from Ru–Ab and QDs is generated. The color of the droplet is thus altered to the mixed color of Ru and QDs. This droplet sensor has some advantages compared to the sensor in tubes: firstly, automation of the droplet platform is realized more easily, which is promising for high throughput assay; secondly, the droplet can enhance the binding probability of protein-protein interactions and accelerate the reaction process due to its ultra-small size; finally, convenient and quick detection methods can be established by the introduction of droplet manipulation technologies. Compared with DeMello's work, this droplet sensor strategy is more suitable for protein study because it is not limited by the protein size. Moreover, the coupling process is relatively less and the experimental process can be recorded by a low-cost imaging device. The use of QDs as a main detection signal can also enhance the sensitivity.

We first evaluated the feasibility of AFP determination by Ru–Ab complex and QDs as the dual fluorescent signals in the macroenvironment. As shown in Fig. 2A, the fluorescence quenching percentage reached at *ca.* 89% with 15 μg ml^{-1} Ru–Ab and 22 nM QDs in the absence of AFP. When 25 ng ml^{-1} AFP was added, a distinct recovery of fluorescence was observed. The feasibility of quenching and recovery experiments was carried out in a droplet. The results are shown in Fig. 2B. First, pairs of droplet containing Ru–Ab and QDs were generated and fused. In the absence of AFP, the Ru–Ab-QDs droplet displayed red (Fig. 2B(a)), which indicated that the fluorescence of QDs had significantly decreased. When AFP was added to the well-mixed Ru–Ab-QDs droplet (Fig. 2B(b)), an orange droplet was observed. The performances shown in droplet agreed with those obtained by spectrometry. This suggests that electrostatic interaction of the reagents and specific affinity of the protein-protein complex can be achieved in a droplet with high and good mixing efficiency. Thus, the design for colorimetric detection of AFP is feasible in a droplet.

3.2 Optimization of variables

In order to obtain high sensitivity, the variables have been optimized. Owing to the weak fluorescence of Ru, its concentration was fixed at 0.3 mg ml^{-1} for effective quenching. Color response of this droplet sensor relies on the restoration of QDs fluorescence, the effect of the concentration of QDs on the quenching and recovery processes was explored and the results

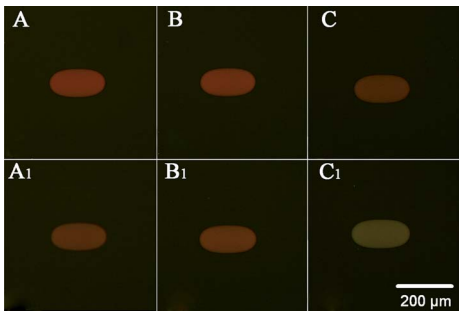

Fig. 3 Effect of concentration of QDs on the quenching and recovery processes. The first and second rows are in the absence and presence of AFP. Concentrations of QDs were (A) 1.4×10^{-6} M, (B) 2.1×10^{-6} M, (C) 3.5×10^{-6} M. Concentrations of Ru–Ab and AFP were 0.3 mg ml^{-1} and 1μg ml^{-1}, respectively.

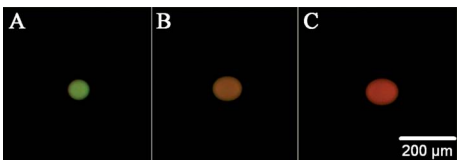

Fig. 4 Effect of volume ratio between QDs and Ru on the quenching process. The volume ratios of QDs/Ru were 1 : 1, 1 : 2 and 1 : 3 from A to C. Concentrations of QDs and Ru–Ab were 3.5×10^{-6} M and 0.3 mg ml^{-1}, respectively.

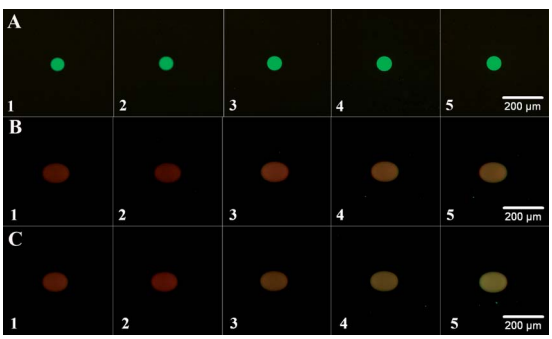

Fig. 5 Color responses at different concentrations of QDs with a fixed concentration of Ru–Ab and VRs of QDs-Ru–Ab. (A) shows the fluorescence of QDs. (B) and (C) show the color response of QDs and Ru–Ab in the absence and presence of AFP. Concentrations of QDs from 1–5 were 1.4×10^{-6} M, 2.1×10^{-6} M, 3.5×10^{-6} M, 5×10^{-6} M and 7×10^{-6} M. Concentrations of Ru–Ab and AFP were 0.3 mg ml^{-1} and 0.01 μg ml^{-1}. The VRs of QDs-Ru–Ab was 1 : 3.

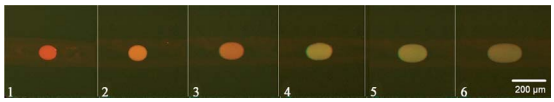

Fig. 6 Effect of the droplet size of AFP on the fluorescence recovery of QDs. Volume ratios of QDs/Ru–Ab/AFP were 1 : 3 : 0, 1 : 3 : 1, 1 : 3 : 2, 1 : 3 : 3, 1 : 3 : 4 and 1 : 3 : 5 from 1 to 6. Concentrations of QDs and Ru–Ab were the same as Fig. 3. The concentration of AFP was 0.01 μg ml^{-1}.

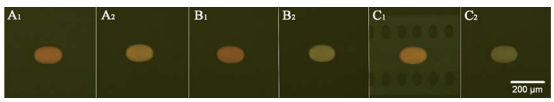

Fig. 7 Comparisons of color change in the absence (left) and presence (right) of AFP under a different mixed order of regeants: (A) QDs-AFP-Ru–Ab, (B) Ru–Ab-AFP-QDs and (C) Ru–Ab-QDs-AFP. Other experimental conditions were kept the same as in Fig. 5.

are shown in Fig. 3. The corresponding data of fluorescence recovery of QDs are presented in Fig. S3.† Evidently, when the concentration of QDs was 3.5×10^{-6} M, the restoration effect was better than in others. This concentration was therefore chosen in the following work. The volume ratio (VRs) between Ru and QDs was investigated. As shown in Fig. 4, the color change appeared when the VRs of QDs/Ru–Ab was up to 1 : 2. However, the VRs of QDs and Ru–Ab was chosen as 1 : 3 for good contrast effect. With higher QDs concentration, however, the fluorescence of QDs could not be quenched completely, leading to a narrower range of color change (Fig. 5). This again showed that 3.5×10^{-6} M QDs was suitable for subsequent experiments. The effect of AFP to droplet size on the fluorescence recovery of QDs was explored in Fig. 6. With VRs of Ru-QDs-AFP up to 1 : 3 : 3, the addition of AFP to QDs/Ru–Ab droplet led to an obvious color change but the recovery effect was poorer when the droplet size of AFP continued to increase, demonstrating that QDs fluorescence recovered to a maximal degree and reached saturation with the VRs of Ru-QDs-AFP at 1 : 3 : 3. The reason for this might be that the concentrations of QDs and Ru–Ab signficantly decreased causing the weak fluorescence signals. Additionally, the effect of mixed order of droplets was investigated on the droplet platform. As shown in Fig. 7 and Fig. S4,† no significant differences in color changes were found in the three modes. The results proved that the chemical reagents mix well within a short time and that the droplet platform is suitable for reactions requiring a long time such as DNA hybridization, enzyme assay *etc.* In summary, the Ru–Ab-QDs-AFP mode was employed with VRs at 3 : 1 : 3 to compare the phenomena in the absence and presence of AFP.

3.3 Specificity of the colorimetric droplet sensor for AFP

To assess the specificity of this colorimetric droplet sensor for AFP, the influences of some biological species including metal ions, amino acids and other proteins were examined in aqueous buffer. The corresponding images and data of fluorescence recovery of QDs are shown in Fig. 8 and Fig. S5.† It is seen that none of these similar proteins caused color variation with concentrations up to 0.1 mg ml^{-1}. Likewise, no detectable color responses were found when amino acids and metal ions were

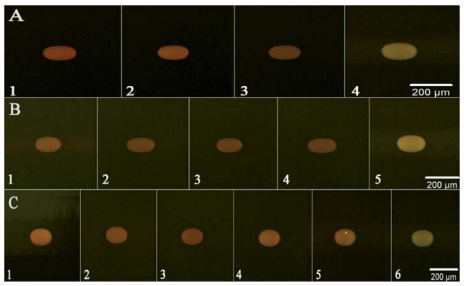

Fig. 8 Color responses of quenched droplets to diverse analytes with (A) HSA, IgG, transferrin (0.1 mg ml^{-1}) and AFP; (B) leucine, isoleucine, methionine, phenylalanine (10 mM) and AFP; (C) buffer, Na^+, K^+, Mg^{2+} (150 mM), Ca^{2+} (1.5 mM) and AFP. Concentrations of QDs, Ru–Ab and AFP were 3.5×10^{-6} M, 0.3 mg ml^{-1} and 0.01 μg ml^{-1} in the system. Other experimental conditions are the same as in Fig. 2.

added to the quenched droplet, only 0.1 μg ml^{-1} of AFP resulted in a significant color change. The specific detection of this biosensor for AFP can be attributed to the following: on the one hand, in the presence of electrolytes and other proteins at high concentrations there is only electrostatic interaction between the QDs or Ru–Ab, causing the electrolytes and other proteins close to QDs or Ru–Ab to form a complex. The quenching between QDs and Ru–Ab is not affected. On the other hand, with the addition of AFP to the QDs-Ru–Ab droplet, the Ru–Ab and AFP first form a whole due to the strong and specific affinity of antigen-antibody. As a result, the repulsive interactions between negatively charged Ru–Ab-AFP and QDs (Fig. S2†) make the Ru–Ab move far away from the surface of QDs, leading to the fluorescence recovery of QDs. Therefore, this strategy demonstrates its specificity in the presence of electrolytes and other proteins at high concentration in the system. Moreover, it can also work well with serum.

3.4 Droplet-based colorimetric homogeneous immunoassay for AFP in aqueous buffer

To further demonstrate the viability of the colorimetric droplet biosensor for AFP detection, different concentrations of AFP were introduced into the sensing platform (Fig. 9). It was seen that with a fixed amount of Ru–Ab, the fluorescence of QDs was quenched in the absence of AFP and the droplet was red in color. With an increase of AFP concentration into the droplet of QDs-Ru–Ab, more and more QDs were released. Correspondingly, a

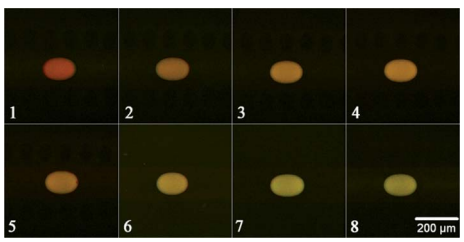

Fig. 9 Color changes of the quenched droplets in the presence of AFP, with increasing concentration of 0, 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05 and 0.1 μg ml^{-1} (from 1 to 8). Other experimental conditions are the same as in Fig. 8.

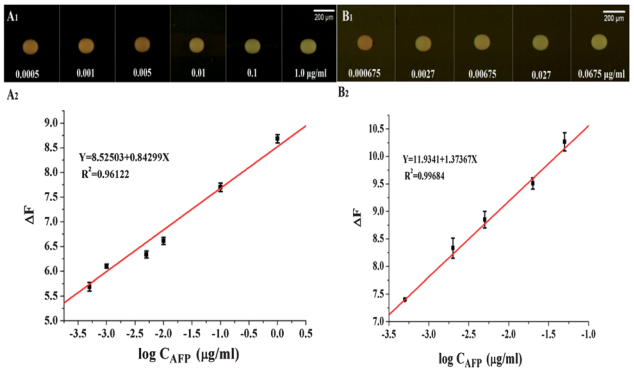

Fig. 10 Relationships between fluorescence restoration (ΔF) and concentrations of AFP (A) in aqueous buffer and (B) in human sera. A1 and B1 present the color responses *versus* the corresponding AFP concentration. The ΔF is the difference in the fluorescence intensity of QDs between AFP droplet and background. Concentrations of QDs and Ru–Ab were 3.5×10^{-6} M and 0.3 mg ml^{-1}, respectively. The average size of a droplet was 0.2 nl. The VRs of QDs/Ru–Ab/AFP were 1 : 3 : 3.

light orange color appeared first, and then gradually deepened, finally turning to green. By comparing the color change of the droplet, at concentrations as low as 0.1 ng ml^{-1} AFP could be identified. The relative standard deviation for 11 replicate measurements of AFP at 0.01 μg ml^{-1} was 2.8%. The fluorescence restoration (ΔF) was linearly related to the logarithm of AFP concentration (log C_{AFP}) over the range from 0.0005 to 1 μg ml^{-1} with a correlation coefficient of 0.9612 (Fig. 10A). The limit of detection (LOD) of AFP was calculated as 0.06 ng ml^{-1}. Thus, the viability of the colorimetric droplet platform for AFP detection has been proven with excellent sensitivity. This platform can also be applied to quantitative assay of other proteins by coupling the corresponding antibody to Ru.

This method and some other reported techniques, that greatly improve sensitivity in the AFP assay, are listed in Table S1.† The sensitivity in this present work was found to be higher than most of the methods listed in Table S1.† Although it is not as sensitive as the results presented in some works, the absolute amount of reagents in our design is smaller than those recorded in the other methods. More importantly, our method does not need time-consuming washing and incubation steps, as well as an additional detection instrument. According to the results obtained, it can be concluded that this method shows excellent prospects for protein detection and clinical diagnosis.

3.5 Application to quantitative analysis of clinical samples

To evaluate the application of this colorimetric droplet sensor for AFP assay in clinical samples, seven human serum samples were diluted and introduced into the platform and the results are summarized in Table 1. According to the color difference of the images between the control and clinical samples, AFP concentration (C_{AFP}) in the clinical samples was easily identified. When there is no or low C_{AFP}, the colors show no obvious differences between controls and clinical samples (A–D). While the C_{AFP} was high, distinct differences were observed (E–G). By comparing the degree of fluorescence recovery of QDs (Δf) between the standard and clinical samples, the C_{AFP} of clinical samples (A–G) were

Table 1 Identification and semi-quantification of AFP in clinical samples

Control	Clinic samples	Control	Standard samples (μg/ml)	C_{AFP} in the clinic samples (μg/ml)
A	Δf=0.42431			< 0.0001
B	Δf=0.42284		0.0001 Δf=2.13650	< 0.0001
C	Δf=0.20459			< 0.0001
D	Δf=2.76792		0.005 Δf=3.11354	0.0001-0.005
E	Δf=2.82241			0.0001-0.005
F	Δf=3.40544		0.01 Δf=4.08105	0.005-0.01
G	Δf=4.64232			>0.01

semiquantitatively determined. As shown in Table 1, the experimental results are in good agreement with the reference value provided by the hospital. Further, a linear range from 0.675 ng ml^{-1} to 0.0675 μg ml^{-1} (Fig. 10B) for AFP with a correlation coefficient of 0.9969 was obtained in the diluted human sera. The LOD of AFP in sera was calculated as 0.4 ng ml^{-1}.

Three independent serum samples were further tested and the results are listed in Table 2. When the level of AFP was over the calibration range, serum samples were appropriately diluted with PBS prior to assay. On the basis of the calibration curve in buffer, AFP concentrations obtained by this colorimetric droplet sensor were close to the reference values provided by the hospital. The results showed an acceptable agreement with the RSD of less than 10%, indicating an acceptable accuracy of the proposed method for the detection of AFP in clinical samples.

Table 2 Analytical results of clinical serum samples by the proposed method

Sample number	Clinical reference value (μg ml^{-1})	Experimental value (μg ml^{-1}) (concentration ± S.D.)[a]	RSD[b] (%) ($n = 3$)
1	0.00260	0.00236 ± 0.00115	9.23
2	0.00650	0.00606 ± 0.000560	6.78
3	0.0260	0.0250 ± 0.0164	3.85

[a] These data are averages from three independent measurements. [b] RSD is the standard deviation of experimental and reference value provided by the hospital.

Therefore, this luminescence-based colorimetric immunodroplet biosensor demonstrates a new prospect of AFP determination in biological samples. It may be suitable as an immunoassay platform for point of care diagnostics because it has high sensitivity, the detection system is low cost, and the detection process is simple, convenient and quick with low consumption of sample and reagent.

4. Conclusions

In conclusion, a new droplet platform applied protein biosensor with fluorescence-based colorimetric detection is presented for the first time. This droplet biosensor for protein detection is not restricted by protein size and so provides a universal detection method for protein assays. The platform has ideal features for practical immunoassay: short assay time, free of washing steps, good selectivity, high sensitivity, low reagents consumption and a low cost detection system. Compared with a DNA droplet sensor, this colorimetric immunosensor can be applied in human serum samples directly. Moreover, this present work provides a sensitive, visual and convenient method to detect large proteins within a droplet. Further development of the droplet technology, offers in the future a strategy with potential in drug discovery, clinical medicine and high throughput biological screening.

Acknowledgements

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