Retraction for *Analyst*:

Quantum dots high fluorescent signal amplification immunoassay using branched DNA and peptide nucleic acid conjugated antibody

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I, Yuan-Cheng Cao, hereby wholly retract this *Analyst* paper for correction. This article was submitted for publication without the knowledge and approval of the co-authors listed.

Signed: Yuan-Cheng Cao, Newcastle University, UK, December 2011.

This retraction is endorsed by May Copsey, Editor. Retraction published 16th December 2011.

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PAPER

Quantum dots high fluorescent signal amplification immunoassay using branched DNA and peptide nucleic acid conjugated antibody

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This study presents a hybrid quantum dots immunoassay using peptide nucleic acid (PNA) and branched DNA (bDNA). First, PNA sequences (5.5 kDa) were conjugated to antibodies through EDC/NHS coupling reagents and the reactions were separated by the molecular weight cut-off ultrafiltration units YM-50 (cut-offs at 50 kDa) and YM-3 (cut-offs at 3 kDa) respectively. UV absorbance was used to calculate the molar ratio of the PNA and antibody involved in the conjugation which was 4. Further mass spectrometry results showed that the Fc fragment ions $[Fc + H]^{1+}$ changed from 48.003 kDa to 72.489 kDa while the Fab fragments remained the same after the papain and pepsin enzyme digestions of the PNA–IgG conjugates. Then the resultant PNA–IgG conjugates were applied to a standard sandwich immunoassay using a quantum dots (QDs) based bDNA high signal amplification system as fluorescent label. The results showed that the sensitivity can be improved by 30 times and the limit of detection (LOD) can be improved by 100 times compared to the direct FITC labelled antibody immunoassay under the same conditions.

Introduction

The quantification of serum virus levels is crucial for monitoring the therapeutic response of chronic infections (such as HIV, HCV).[1–6] Currently, there are a variety of assays available for monitoring viral infections in serum at DNA/RNA or antigen/antibody levels. The bDNA (branched DNA) assay method is well known to have a wide detection range and a low detection limitation among all the diagnostic methods.[2–8] For example, the liquid hybridisation has a detection limit of 1–2 pg mL^{-1} compared to 2.45 pg mL^{-1} for the bDNA method.[6–8] The bDNA assay showed excellent reliability, returning a negative result for 99.7% of the sera specimens from healthy blood donors.[7] However, the standard bDNA detection is really time-consuming and the applications are limited to the nucleic acid (RNA or DNA) based detections; and it has not been applied to detect the immune response products such as antigens or antibodies.

Synthetic peptide nucleic acid (PNA) has been used in many fields such as molecular biology procedures, diagnostic assays and antisense therapies.[9–15] Different from the DNA and RNA, the backbone of PNA contains no charged phosphate groups and therefore, it makes the binding between PNA/DNA strands stronger than between DNA/DNA strands due to the lack of electrostatic repulsion. And also, PNA oligomers show greater specificity in binding to complementary DNA, with a PNA/DNA base-pair being more stabilizing than a similar match in a DNA/DNA duplex.[9,10] The main concern of the length of the PNA-oligomers is to guarantee specificity. As PNA is the DNA analogue which has resistance to most of the proteases and nucleases, it is stable in the detections aimed at the samples which contain the enzymes. PNA sequences are also stable over a wide pH range. Therefore, PNA is an ideal choice for creating a hybrid bDNA–protein immunoassay by conjugating the PNA to an antigen or to an antibody raised against a particular virus, and so the bDNA can be applied to detect the presence of antigen or antibody in an immunoassay.

Meanwhile, quantum dots (QDs), the colloidal semiconductor nanoparticles with unique luminescence characteristics, have attracted extensive attention as novel fluorescence indicators in numerous biological processes and bio-analysis recently.[16–22] The unique optical and electronic properties of QDs such as wide and continuous absorption spectra, narrow emission spectra, and high light stability are more attractive than the traditional dyes.[20–22] For example, a QD as a fluorophore is 20 times brighter than the RhB single molecule.[22] The remarkable size-dependent properties of QDs have turned them into promising materials for many applications. And therefore, QDs have been widely used in various fluorescence-based sensors for the detection of specific analytes.

In this work, the bDNA analytical system was applied to the immunoassay system by introducing PNAs which conjugated with the antibody. QDs as a bright fluorophore were used as the fluorescent reporter for the bDNA signal amplification. The analyte in the immunoassay was captured by the PNA conjugated antibody and PNAs were recognized by the branched

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DNA where the analyte concentration information was transferred to the QDs fluorescence signal, which achieved high fluorescent signal amplification. This method achieved a high fluorescent/protein (*F*/*P*) ratio for the immunoassays by introducing branched DNA and bright photo-robust fluorophore QDs. This should result in more sensitive detection than the directly labelled antibody related immunoassays and it also extends the bDNA application to the immunoassay systems.

Experimental

Reagents and chemicals

Mouse anti-human IgG, human IgG and rabbit anti-mouse IgG were obtained from Sigma; pepsin (P6887) and papain from papaya (P3125) were purchased from Sigma; ultrafiltration units were obtained from Millipore ultrafiltration products (Centriprep Ultracel YM-3 and YM-50; catalogue no. UFC800324 and UFC805024, respectively); the optical bottom 96-well plate used in this work was Nunc™ 96 MicroWell (catalogue 164587, Nalge Nunc International, USA) and avidin modified CdSe@ZnS quantum dots (QDs) were purchased from Evident Technologies; peptide nucleic acid (PNA, $M_w = 5476.49$ Da) was obtained from Biosynthesis Inc. which was functionalized with an $-NH_2$ terminal and the space arm at the 5′ end; the branched DNA (bDNA) reagent (QuantiGene 2.0) set was purchased from Panomics Inc.

PNA and antibody conjugation

$-NH_2$ terminal modified PNA and rabbit anti-mouse antibody conjugation experiments were carried out by the $-NH_2$ and –COOH ester reaction in the presence of coupling reagents EDC/sulfo-NHS according to ref. 17 which is illustrated in Fig. 1. In brief, rabbit anti-mouse IgG solution of 600 μg mL^{-1} and PNA of 200 μg mL^{-1} were prepared first; then 15 mL PNA solution and 15 mL IgG solution were mixed before adding the freshly made EDC/sulfo-NHS solution; the final concentrations in the mixture were: PNA 100 μg mL^{-1} (18.5 nmol mL^{-1}), IgG 300 μg mL^{-1} (about 2 nmol mL^{-1}), EDC 60 μg mL^{-1} and NHS 10 μg mL^{-1}.

Two parallel experiments and a control experiment were carried out under the same conditions. For the control reaction, the experiment was carried out without the addition of EDC/NHS during the conjugation. The mixture was shaken for 4 hours at room temperature before the ultrafiltration. All the conditions were the same.

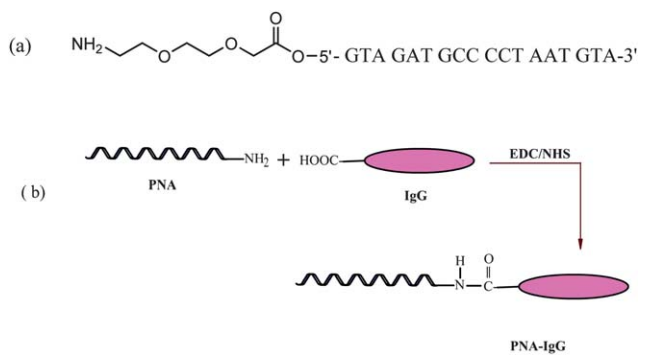

Fig. 1 PNA probe molecular structure (a) and the illustration of PNA sequence and antibody IgG protein conjugation (b).

Ultrafiltration

Millipore ultrafiltration units YM-3 with a molecular weight cut-off (MWCO) of 3 kDa, and YM-50 with a MWCO of 50 kDa were used in order to separate the free PNA molecules from the reaction solution and desalt the samples as well. In brief, first, the sample was injected into the YM-50 unit, 5 mL each unit; and then centrifuged at 4000 rpm for about 45 min (there was about 0.5–1.0 mL solution left in the resident solution) and then 5 mL distilled water was added to the resident solution which was followed by centrifugation and this washing process was repeated for 3 times. All the filtered solution which contained the free PNA and salts was collected; then PBS was used to dilute the resident solution to 10 mL and this resident solution (*YM-50 Resident*) contained the conjugated PNA–IgG which was then kept in a fridge for UV, MS and immunoassays. Second, YM-3 was used to filter the solution that was collected from the YM-50 (*YM-50 Filtered*), and centrifuged at 4000 rpm for about 1 hour (there was about 1 mL solution left). Then 10 mL distilled water was added and the unit was centrifuged again. This washing process was repeated for 3 times and then dilute the retained solution to 10 mL which only contained free PNA (*YM-3 Resident*). This process is shown in Fig. 2. The reacted PNA in the conjugation experiments was calculated from the standard absorbance curves.

Enzyme digestion

First, papain was dissolved in 0.1 mol L^{-1} sodium acetate, pH 5.5 with 3 mmol L^{-1} EDTA, and then it was incubated for 30 min at 37 °C on the shaking plate to preactivate the enzyme. Then the papain solution was diluted to 2 mg mL^{-1}. The preactivated papain was added to the sample (IgG or PNA–IgG) at a ratio of 1 : 20 (w/w) and the mixture was incubated at 37 °C for 2 hours with the shaking plate. In theory, the IgG should be digested into Fab and Fc fractions during this enzyme digestion.

Pepsin was dissolved in sodium acetate at 50 μg mL^{-1} and the pH was adjusted to 4 by using 1 N HCl and sodium carbonate solution. 20 μL freshly made enzyme solution was mixed with the 0.5 mL sample (rabbit anti-mouse IgG and PNA–IgG respectively). The tubes were sealed and kept shaking at 37 °C overnight. After that, sodium carbonate solution was used to adjust the pH value to 7.5 to inactivate the enzyme. Then the digested samples were loaded to YM-3 to desalt before the mass spectrometry test.

MALDI-mass spectrometry test

The samples after the enzyme digestion were applied to the Matrix Assisted Laser Desorption Ionization Mass Spectrometry, namely MALDI-MS (Shimadzu, Kratos Axima), sampling plate and then the sampling plate was dried under the lamp for about 30 min. Before the measurements three proteins from *laserbiolabs* were used for calibration in these experiments, *i.e.* insulin oxidised B chain, horse myoglobin, and trypsinogen.

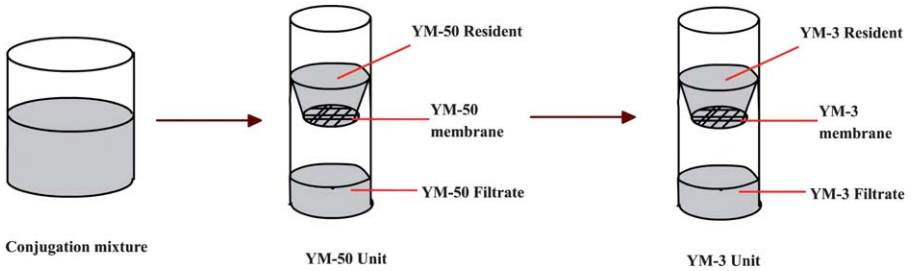

Fig. 2 Illustration of the ultrafiltration separation process for the conjugation.

Plate preparation for immunoassays

Human IgG in 100 μg mL^{-1} was applied to the 96-well plate at the loading volume of 50 μL per well and the plate was incubated at room temperature overnight with the shaking plate followed by blocking with 0.5% casein which was used as blocking reagent during this immunoassay. After this, several rounds of PBS washing were applied to wash the wells. And then mouse-anti-human IgG which acted as the analyte molecule in a series of concentrations was applied to the wells, again the plate was incubated on the shaking plate which was followed by 3 times of PBS washing of each well. The wells without analyte were used as control wells (blank) and all the other conditions were the same. And then the plate can be used for the FITC direct labeled anti-mouse antibody immunoassays. For the PNA–bDNA immunoassays, 20 μg mL^{-1} PNA conjugated rabbit anti-mouse IgG was loaded to the wells (10 μL each well) and incubated on the shaking plate for 4 hours which was followed by PBS washing 3 times.

bDNA fluorescent immunoassays

After the plate preparation step, FITC direct labeled anti-mouse antibody was used as fluorescent label following the standard immunoassay procedures for the detection performance comparison and at the same time, bDNA reagents were applied for the PNA–bDNA based immunoassay using QDs as fluorescent label. For the PNA–bDNA immunoassay, the experiments were carried out step by step according to the QuantiGene 2.0 handbook. Briefly, first, pre-amplifier was loaded to the plate prepared as above to hybridise the PNA sequence which took about 1 hour at 55 °C; and then PBS was applied to wash the wells 3 times. Second, 100 μL of QuantiGene 2.0 amplifier working reagent was added to each well of the plate and the plate was sealed at 55 °C for 1 hour to hybridize the amplifier, and this was followed by PBS wash 3 times. Then, streptavidin coated CdSe@ZnS QDs which were diluted in Label Probe Diluent were added to the wells at 100 μl per well, and the plate was sealed at 35 °C for 1 hour on the shaking plate before using the microscope to detect the fluorescent signal.

Characteristic

In the fluorescent detection experiments, the microscope (Olympus IX-71, Japan) was linked with the camera (PCO1600, PCO, GMbH, Germany) and an imaging spectroscope (PARISS, Lightform Inc., USA). A mercury laser was used as the light source. FITC and QDs fluorescent spectra were detected with a filter set consisting of a 480–550 nm band-pass (BP) excitation filter, 570 nm dichroic mirror (DM) and 590 nm long pass (LP) emission filter (U-MSWG2; Olympus).

Results and discussion

PNA and antibody conjugation

Due to the various functional groups such as –COOH, –SH and $–NH_2$, IgG is easy to be further modified with special probes in different applications.[17] In the meantime, peptide nucleic acid (PNA) is a unique probe which has been used in many fields due to the unique properties compared to DNA or RNA[9,10] such as the high binding strength and specificity. In most of the applications, 18–25 base strands of PNA sequence are sufficient for the reactions. And due to their higher binding strength it is not necessary to employ long PNA oligomers for the uses in the duplex reactions.[9] The main concern of the length of the PNA-oligomers is to guarantee the specificity. In this work, an 18-base single-strand PNA probe, designed with the modified $–NH_2$ group at the terminus and with –O– bridges to increase the solubility (Fig. 1a), was covalently conjugated with the IgG under the coupling reagents EDC/NHS and the process is shown in Fig. 1b.

As the typical IgG molecular weight is around 150 kDa while the PNA is 5.5 kDa, molecular weight selective ultrafiltration membranes can be employed to separate these two types of molecules and also desalt the reaction solutions due to the molecular weight difference. This separation process is shown in Fig. 2. First, YM-3 ultrafiltration units (M_w cut-off at 3 kDa), which can allow the molecule with $M_w < 3$ kDa to pass through while stopping the molecule with $M_w > 3$ kDa, were tested to separate the PNA from solution. YM-3 filtration performance test results showed that the upper retained solution had the higher UV absorbance value compared with the original solution while the filtered solution showed ignorable absorbance at 280 nm (Fig. 3a). The same tests showed that the absorbance values from the upper retained solution and the filtered solution were the same after passing through the YM-50 ultrafiltration units (M_w cut-off at 50 kDa) (Fig. 3b). These results indicated that the YM-3 can separate the free PNA from solution effectively while the YM-50 showed no effects against PNA.

During the separation process after the conjugation, YM-50 was first used to separate the PNA–IgG complex (in the upper retained solution) and then YM-3 was used to separate the free PNA, and at the same time desalt the conjugate reagents such as EDC and NHS. The filtration performance results of the

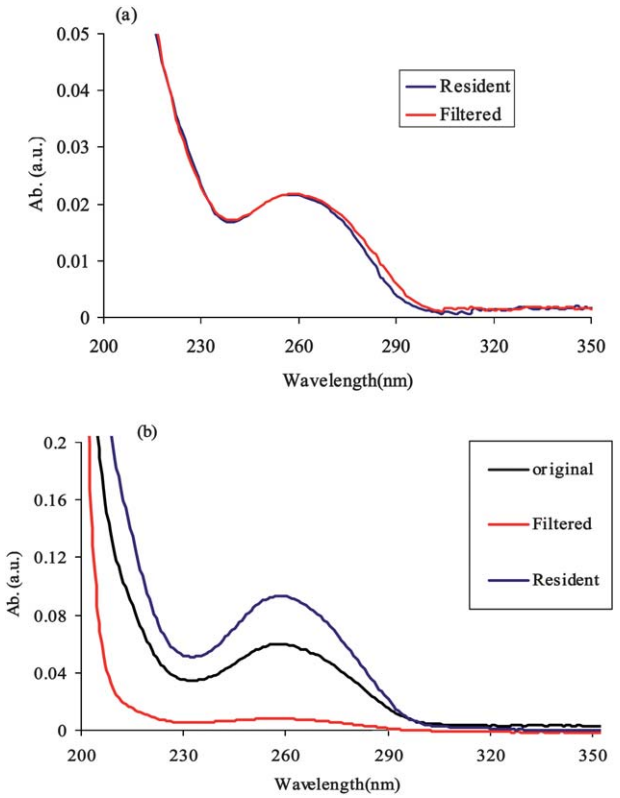

Fig. 3 Filtration performance test of the ultrafiltration units against PNA. UV absorbance of the reaction starters by the ultrafiltration units YM-50 (a) and YM-3 (b). 10 μg mL^{-1} PNA was used as the original solution before the ultrafiltration.

ultrafiltration unit YM-50 against the conjugation solution showed that the upper retained solution had quite strong absorbance at 280 nm which represents the IgG maximum absorbance while the filtered solution had the maximum absorbance at 260 nm which represents the PNA absorbance (Fig. 4). These results indicated that the combination of the ultrafiltration units can separate the free PNA and conjugation resultant, namely the PNA–IgG complex, successfully.

In order to confirm whether the reaction is covalent conjugation or physical absorption between the PNA and IgG, two

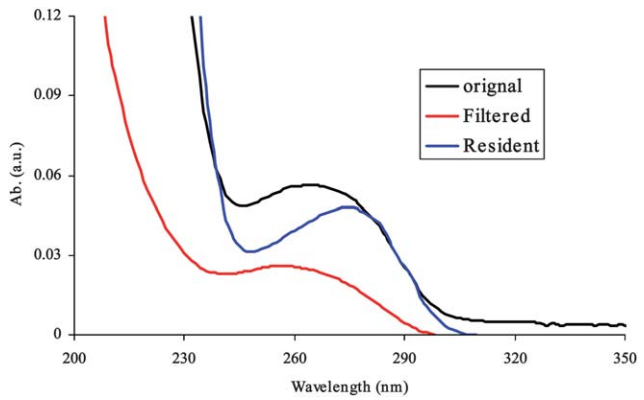

Fig. 4 Filtration performance of the ultrafiltration unit YM-50 against the conjugation solution.

Table 1 IgG fraction UV absorbance (λ260/280) after ultrafiltration through YM-50/YM-3

Experiment	IgG	PNA–IgG fraction	PNA
+EDC/NHS	0.6085	1.4098	1.7363
−EDC/NHS	0.6058	0.6895	1.7363

parallel experiments were carried out: one with coupling reagents EDC/NHS (+EDC/NHS) and another without coupling reagents (−EDC/NHS). By measuring the individual λ260/280 ratio of IgG, PNA and PNA–IgG fractions from the parallel experiments, the results (Table 1) showed that the upper retained solution from the reaction with coupling reagents had much higher ratio (λ260/280 = 1.4098) than the experiment without coupling reagents (λ260/280 = 0.6895), which means that there were more PNA molecules attached to the IgG when coupling reagents were added during the conjugation, while the λ260/280 ratio for pure IgG is 0.6085 and PNA is 1.7363 (this difference is due to the different maximum absorbance). These results indicated that the reaction with coupling reagents is a covalent reaction and EDC/NHS plays a coupling reagent role in the conjugation reaction.

Since the reacted PNA in the conjugation process can be calculated from the standard absorbance curves, the PNA/IgG molar ratio can also be calculated when a fixed amount of antibody was used in the experiments. In order to quantify the conjugation between the PNA probe and antibody IgG, the UV absorbance measurements were carried out before and after the reaction during the filtration. By the calculation (Table 2), the conjugated PNA/IgG molar ratio was about 4, which means that each of the antibodies has 4 PNAs attached on average.

PNA–IgG molecule structure confirmation

In order to further confirm if the antibody has the same number of attachments and where these conjugation sites are on the antibody, enzyme digestion experiments and mass spectroscopy were carried out after the conjugation and separation. Papain and pepsin are two commonly used enzymes to study the protein structures and sub-units.[23–28] Here we applied these two enzymes to study the conjugation sites of the PNA sequences on the antibody.

After the enzyme digestion, MALDI-TOF MS was applied to study the fragments of the resultants and the spectra results showed that the peaks corresponding to the ions $[IgG + H]^{1+}$ and $[IgG + 2H]^{2+}$ for the whole rabbit anti-mouse IgG were observed (Fig. 5a). From the results we can see that the molecular weight of rabbit anti-mouse IgG was 146.4772 kDa;

Table 2 The molar ratio of the PNA/antibody

Repeatant	IgG/ nmol mL^{-1}	ΔPNA		PNA/IgG molar ratio
		μg mL^{-1}	nmol mL^{-1}	
1	2.05	45.5325	8.320	4.01
2	2.05	46.0605	8.417	4.06

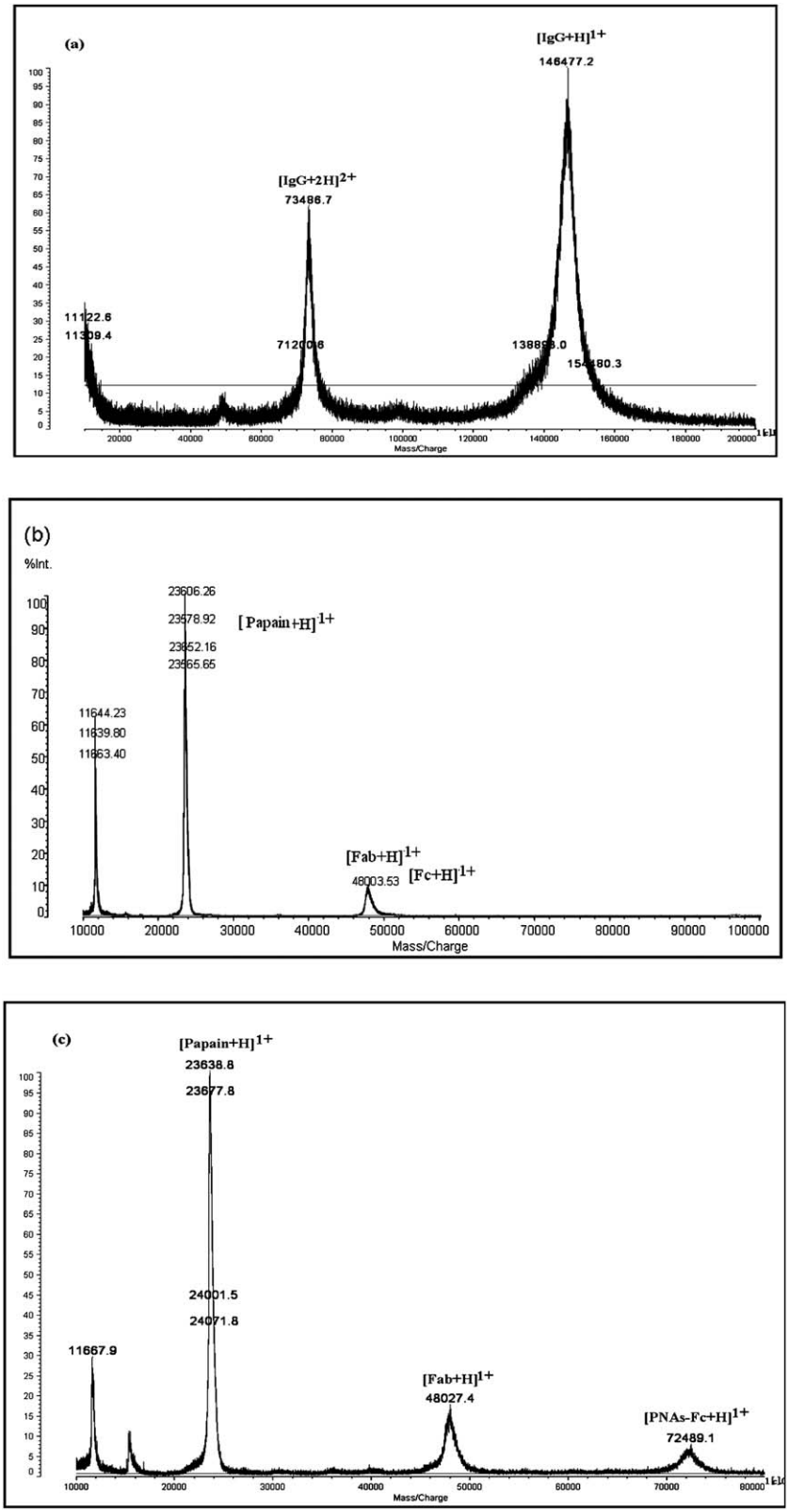

Fig. 5 MALDI-MS spectra of the IgG (a), the digested fragments of IgG (b) and the digested PNA conjugated IgG (c).

and papain enzyme digestion results showed that the whole antibody molecule can be digested into two fragments, namely Fab and Fc, and the ions corresponding to these two peaks were 47.896 kDa and 50.8892 kDa respectively in the MS spectrum (Fig. 5b); while for the digestions of PNA conjugated antibody, the ions $[Fab + H]^{1+}$ and $[Fc + H]^{1+}$ were detected at 48.0274 kDa and 72.4891 kDa respectively (Fig. 5c). The only difference in MS is that the ion for $[Fc + H]^{1+}$ was shifted about

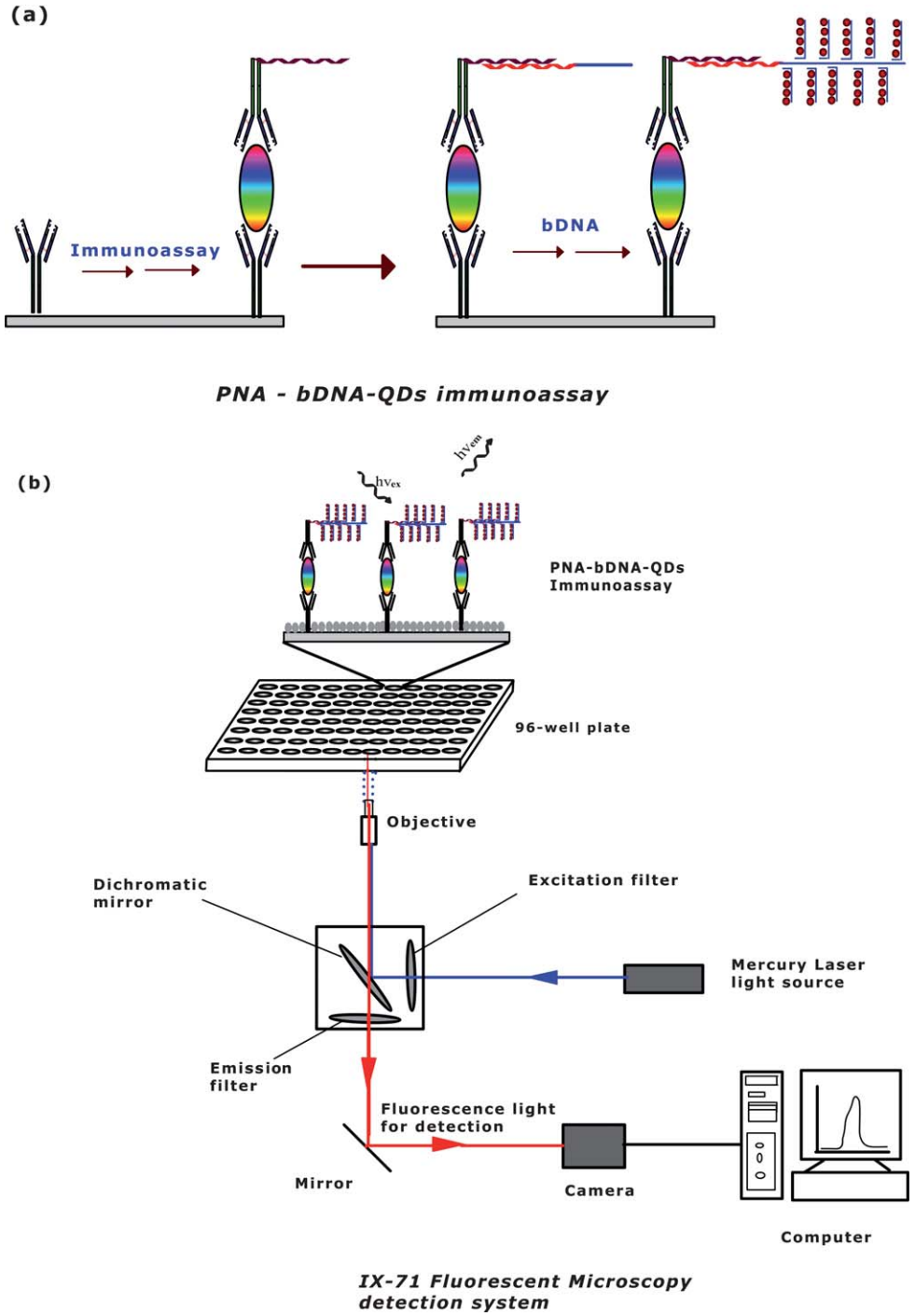

Fig. 6 Illustration of PNA–IgG related bDNA high signal amplification immunoassays (a) and the fluorescent detection system using the IX-71 fluorescent microscope (b).

21 599.9 Da while the $[Fab + H]^{1+}$ was almost the same. As the PNA molecular weight is 5476.49 Da, the whole weight difference on ions $[Fc + H]^{1+}$ should be contributed by four sequences of PNA.

Further experiments showed that the pepsin could digest IgG into several fragments while PNA conjugated IgG could not be digested by this enzyme, which indicates that there might be one or more PNA conjugated near the hinge region hence blocking the pepsin enzymatic sites.

bDNA immunoassays based on PNA–IgG

Further analytical performance tests were carried out using this PNA–IgG complex in standard sandwich immunoassays on the 96-well plate. As the PNA sequences were attached to the Fc region of the IgG, this conjugation will not affect the IgG function in immunoassays, as the antigen recognition sites are located in the Fab region of the antibody. Therefore, this PNA–IgG complex can be applied to the high signal

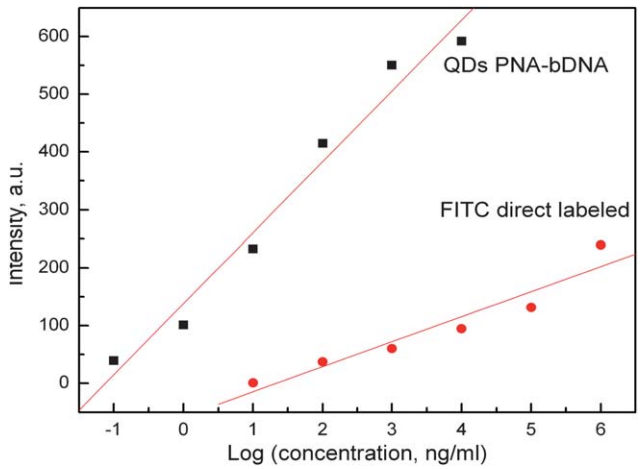

Fig. 7 Dose–response curves of PNA–bDNA immunoassay using QDs and FITC direct labeled immunoassay.

amplification immunoassays in which the PNA is linked with the branched DNA so that the analyte signal in the immunoassays can be amplified by the bDNA–QDs system. The illustration of this immunoassay is shown in Fig. 6a and the fluorescent detection system using the IX71 microscope is shown in Fig. 6b.

High luminescence QDs were used as fluorescent labels in these experiments and for comparison, FITC direct labelled antibody based immunoassays were also performed under the same conditions and the results are shown in Fig. 7. From the results we can see that the sensitivity of the QDs PNA–bDNA immunoassays was nearly 30 times higher than that of the FITC direct labelled immunoassays by calculating the slope of the dose–response curves when the exposure time was taken into account. The limit of detection (LOD) of the QDs PNA–bDNA based immunoassays reached a concentration as low as 10^{-1} ng mL^{-1} while the FITC direct labelled antibody immunoassays could only reach 10 ng mL^{-1} under the same conditions, which means that the LOD of QDs PNA–bDNA immunoassays was more than 100 times lower than that of the FITC direct labelled antibody immunoassays.

Conclusion

Peptide nucleic acid (PNA)–branched DNA (bDNA) can be successfully conjugated to antibodies through EDC/NHS coupling reagents and the reactions can be efficiently separated by the molecular weight cut-off ultrafiltration units 50 kDa and 3 kDa. The molar ratio of the PNA and antibody in the resultant conjugation was calculated to be 4 on average, and the PNA sequences were covalently attached to the Fc fragments of the IgG. This resultant PNA–IgG conjugate can be applied to bDNA based high signal amplification immunoassays using quantum dots (QDs) as fluorescent label which showed that the sensitivity can be improved and the limit of detection (LOD) can be improved as well. This method extends the power of bDNA methods to immunoassays. This study can provide a great approach for the high sensitivity and low analyte analysis applications.

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