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Nonlinear hybridization chain reaction-based flow cytometric immunoassay for the detection of prostate specific antigen

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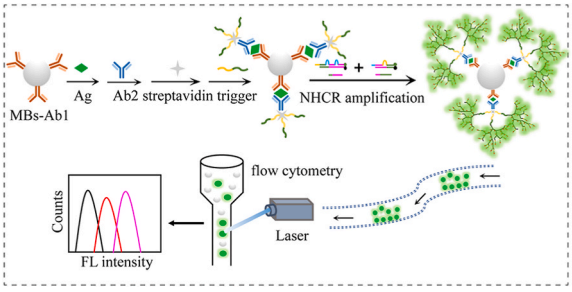
HIGHLIGHTS

- A novel sandwich immune-NHCR assay was developed to achieve highly sensitive detection of prostate-specific antigen.
- High selectivity were ensured *via* NHCR signal amplification with magnetic enrichment.
- Flow cytometry enables direct analysis of NHCR products on each magnetic bead.
- This method was proven to be robust for the detection of human serum.

GRAPHICAL ABSTRACT



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ABSTRACT

Sensitive detection of biomarkers is highly desirable for disease diagnosis and postoperative examination. As a signal amplification technique, nonlinear hybridization chain reactions (NHCR) based on DNA self-assembly has been widely adopted to versatile biosensor platforms for signal output and sensitivity enhancement. Herein, we proposed a novel hairpin-free NHCR based flow cytometric immunoassay for prostate specific antigen (PSA) detection. In this study, Ab1-Ag-Ab2-streptavidin-trigger DNA complexes were formed on the magnetic beads (MBs), and each trigger DNA initiated a round of NHCR amplification to form dendritic DNA nanostructures with many fluorescent signal molecules. The robust flow cytometric fluorescent analysis was finally employed for the quantitation of target protein on the MBs. As far as we know, this is the first time to combine the hairpin-free NHCR strategy with fluorescent immunoassay on MBs to detect protein biomarkers. In addition to the high selectivity of immunoassay itself, the characteristics of isothermal, enzyme-free, and exponential amplification efficiency of hairpin-free NHCR endow this developed immunoassay with a detection limit that exceeds 100-folds that of commercially available PSA kits. Moreover, this MBs-based platform of this immunoassay is also amenable to target enrichment and removal of spontaneous NHCR signal through magnetic separation, greatly eliminating the background signal interference. With our efforts, this newly developed biosensor exhibits a

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detection limit of 1.92 pg/mL and shows high selectivity. It has also been successfully applied to the quantitative detection of PSA in serum samples. With these merits, this convenient biosensor platform has the potential for medical research and disease diagnosis.

Accurate and sensitive detection of biomarkers with facile platforms is essential in bioanalysis and disease diagnosis [1]. Immunosensor is a powerful analytical device that can convert biological signals into other easy detectable signal by integrating with versatile signal transducers [2] (*e.g.* electrochemical, fluorescence, and chemiluminescence). The three core components of the immunosensors are the recognition, amplification, and transduction elements. The high specificity of antigens and antibodies is still regarded as a predominant method in the biomarker recognition step [3,4]. Due to the low abundance of target proteins and the complexity of the blood matrix environment, it is extremely important to develop effective and formidable signal amplification methods. Traditional immunoassays, such as enzyme-linked immunosorbent assay (ELISA) [5] and enzyme immunoassay (EIA), can only provide compromised sensitivity which no longer meets the requirements of point of care testing (POCT) [6]. Besides, the enzyme-based signal amplification method has a slow reaction speed and is easily affected by the environment and temperature.

In recent years, nucleic acid-based signal amplification technology has developed rapidly to improve the sensitivity of immunosensors. Compared with the other common signal amplification technology, nanomaterial-based methods [7–13], nucleic acid-based signal amplification technology has the advantages of low cost, simple operation, and programmability. Immune-polymerase chain reaction (Immune-PCR) [14], uses nucleic acids instead of enzymes as markers, to achieve exponential amplification of DNA molecules through PCR, which enables quantitative detection of antigens with 1000-fold greater sensitivity than traditional ELISA. However, the complex multi-step protocol increases analysis time, and the thermal cycler places high demands on the instrument. Under such circumstances, isothermal amplification strategy has emerged to be an alternative way for signal amplification, such as rolling circle amplification (RCA), loop-mediated isothermal amplification (LAMP) [15], and nucleic acid sequence-based amplification (NASBA) [16]. In particular, hybridization chain reaction (HCR), which is an enzyme-free isothermal amplification strategy developed by Dirks and Pierce [17], has been widely applied to nucleic acids, cancer cells, and proteins detection [18–22]. In this method, the introduction of initiator sequence triggers the polymerization of nicked DNA nanowires through strand displacement between two hairpin structures, HCR is performed under an enzyme-free and thermal cycle-free condition. Nevertheless, traditional HCR still confronts serval problems, such as its linear growth of final products and slow kinetics limiting the detection sensitivity in clinical diagnosis [23,24]; the background leakage still exists in the absence of initiator due to the spontaneous opening of the two hairpin probes [25]. Nonlinear hybridization chain reaction (NHCR), as a newly emerged isothermal amplification strategy, has demonstrated ultra-high growth kinetics through the formation of hyperbranched or dendritic nanostructure [24,26]. This higher dimensional HCR strategy has proven to be effective for a wide range of applications in bioimaging and biosensing [27–29]. Although this hairpin-free NHCR strategy has been mainly combined with electrochemistry, electrochemiluminescence, surface plasmon resonance and fluorescence analysis to detect nucleic acid and ATP, but there is almost no research on the detection of protein based on this hairpin-free NHCR [30–33]. It is very meaningful to develop a method based on hairpin-free NHCR strategy to detect protein.

Prostate cancer is the most common malignant tumor in men worldwide [34]. Prostate-specific antigen (PSA) is widely accepted as a tumor marker in the early diagnosis and prostate cancer is highly suspected when the PSA level is higher than 4 ng/mL. Currently, the main commercial PSA assays include enzyme-linked immunoassay (ELISA) and chemiluminescence immunoassay (CLIA) are well established for the daily diagnosis of PSA. These methods are mainly performed by coating the antigen on a microtiter plate and adding peroxidase to catalyze the substrate reagents to produce fluorescence or chemiluminescence. However, the drawbacks of traditional PSA detection assays are also obvious, such as low sensitivity, lack of automatic detection, and inefficient enzymatic reactions. Especially when diagnosing recurrence of radical prostatectomy (RP) patients, the PSA index is usually as low as fg/mL, and the existing immunodiagnostic kits cannot meet this diagnostic demand [35]. Therefore, the highly sensitive and efficient detection of PSA in serum is profound significant for early diagnosis and prognosis; and the possibility of false positive and negative results also requires a wide dynamic range of quantitative PSA detection methods [36].

To overcome the above obstacles, a novel sandwich PSA immunosensor was developed through the dendritic self-assembly of hairpin-free NHCR and antigen-antibody biomolecular recognition. In our study, the MBs acted as solid phase for the capture antibody (Ab1) immobilization. In the presence of the target antigen, a sandwich-like complex (Ab1-antigen-biotinylated Ab2) was formed *via* specific recognition. Streptavidin (SA) was then introduced as a bridge to connect biotinylated Ab2 and biotinylated trigger DNA, thus triggering the nonlinear HCR process to propagate DNA dendrimer on the MBs surface, generating a detectable fluorescence signal. This developed immunosensor not only owns high selectivity through the specific reaction between antigens and antibodies but also has a low detection limit due to signal amplification of NHCR and enrichment of target antigen by MBs. In addition, the background signal of spontaneous reaction would be eliminated through the elution step of magnetic separation, which greatly improved the sensor efficiency. The enzyme-free environment of biosensing makes it possible to have higher practicability in complex environments. Generally speaking, the biosensor developed by us based on NHCR strategy and immunoassay on MBs can be a promising biosensor with low detection limit, high selectivity, low background, stability, reliability, strong practicality and versatility.

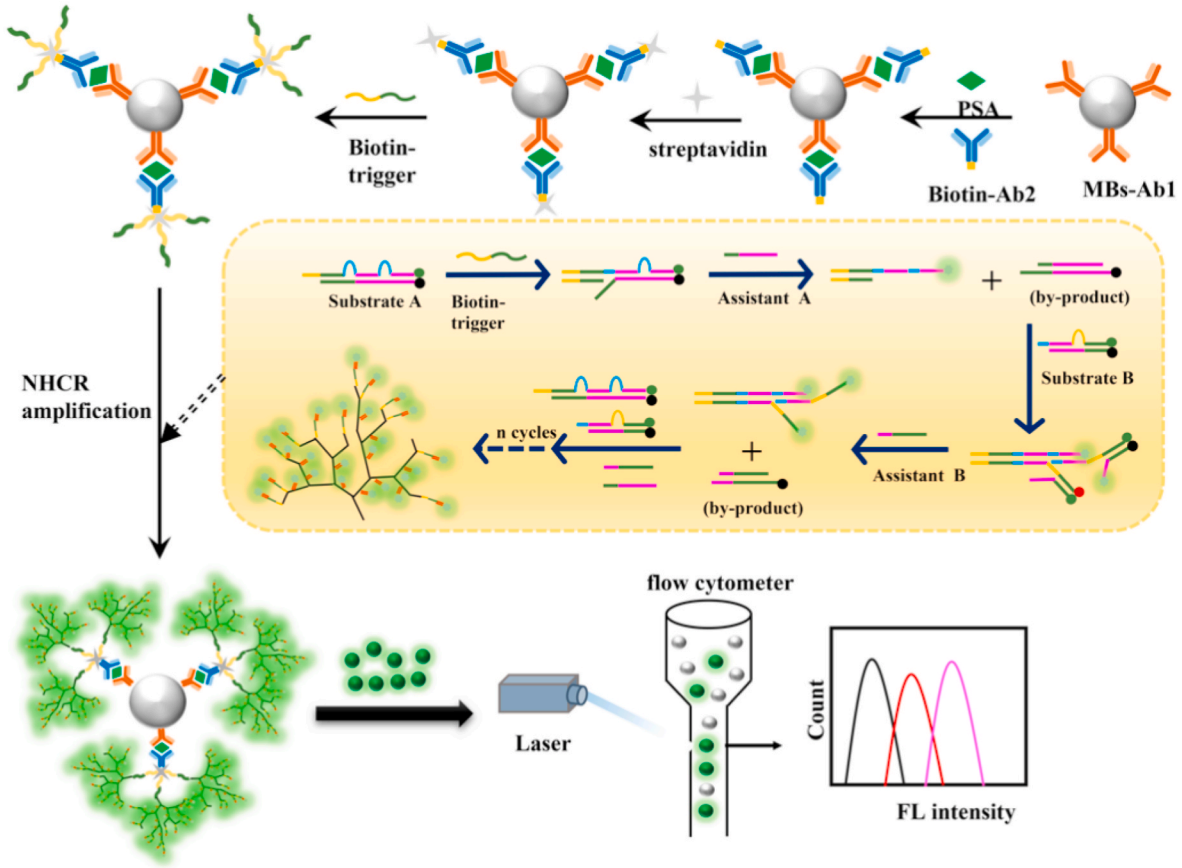
1. Experimental section

1.1. Materials and reagents

Carboxylate MBs (diameter in 3 μm) were purchased from Knowledge & Benefit Sphere Tech. Co., Ltd. (Suzhou, China). Prostate Specific Antigen (PSA) and Monoclonal Antibody to PSA were obtained from Shanghai Linc-Bio Science Co. Ltd. (Shanghai, China). Carcinoembryonic antigen (CEA), alpha fetoprotein (AFP), and immunoglobulin G (IgG) were obtained from Shanghai Linc-Bio Science Co. Ltd. (Shanghai, China). N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 2-[N-morpholino] ethanesulfonic acid (MES), and bovine albumin (BSA) were obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Streptavidin was purchased from Beijing Bioss Biotechnology Co., Ltd. (Beijing, China). Phosphate-buffered saline (PBS; 0.01 M, pH 7.4) was obtained from Thermo Fisher Scientific (sunnyvale, CA). 2-Amino-2-(hydroxymethyl)-propane-1,3-diol (Tris base) was purchased from Biofroxx company (Einhausen, Germany). All oligonucleotides were obtained from Sangon Inc. (Shanghai, China).

1.2. Apparatus

Biometra gradient PCR instrument (Biometra, Goettingen, German),



Scheme 1. Schematic representation of the NHCR based fluorescence immunoassay for the detection of protein biomarkers.

ChemiDoc XRS + molecular imager (Bio-Rad, Hercules, CA), f-7100 fluorescence spectrophotometer (Hitachi, Tokyo, Japan), flow cytometer LSRFortessa (BD Biosciences, San Jose, CA), AFM Bruker Multimode 8 (Bruker, Santa Barbara, CA), DYY-C electrophoresis analyzer (Liuyi Instrument Company, Beijing, China), WH-986 rotary mixer (Qilinbeier, Haimen, China), and PHS-3E pH meter (INESA, Shanghai, China) were applied in this study.

1.3. Preparation of MBs-Antibody conjugates

According to the two-step protocol provided by the manufacturer, 1000 μg magnetic carboxylate beads were washed twice with 0.05 M MES and then activated with 8 μL 50 mg/mL EDC and 5 μL 50 mg/mL NHS. The activated MBs were subsequently washed twice with 0.05 M MES; 250 μg MB were taken out and then incubated with 50 μL 400 μg/mL capture antibodies (Ab1) at room temperature for 2 h. The unreacted antibodies were removed by magnetic separation. The MBs-antibody complexes were then blocked with blocking buffer (5% BSA) at 4 °C overnight. After four washing steps with PBST to remove the residual blocking buffer, the MBs-antibody complexes were stored at 4 °C for further use.

1.4. Preparation of NHCR probes

The DNA oligonucleotides were dissolved in NHCR buffer (1 × TAE buffer supplemented with 12.5 mM Mg^{2+}, pH 8.0). Substrate A and Substrate B were prepared by heating the mixture of F chain (1 μM) and Q chain (1.5 μM) to 95 °C for 5 min, and then cooling to room temperature slowly (1 °C/min). Each substrate chain was mixed with the corresponding Assistant A/B chain (2 μM) to ensure that the excess Q chain was removed *via* by-products forming process. The prepared probes are stored in a 4 °C refrigerator for later use.

For the fluorescence analysis, NHCR probes (Substrate A: Substrate B = 1:2), and trigger DNA with different concentrations were mixed and incubated at 37 °C for 1 h, giving the final concentration of NHCR probes of about 0.15 μM Substrate A, 0.3 μM Substrate B, 0.23 μM Assistant A, and 0.46 μM Assistant B. The real-time fluorescence measurements were performed using f-7100 fluorescence spectrophotometer with excitation at 490 nm and emission at 518 nm.

1.5. Analytical process

After preparation of MBs-antibody conjugates, take MBs-antibody complexes 10 μg for each group, 1000 μL different concentration of PSA antigen (from 0–1000 pg/mL, a total 10 groups) was added into the MBs-Antibody conjugates and incubated for 2 h at room temperature. Then, 200 μL 1 μg/mL biotinylated detection antibody (Ab2) was introduced and incubated for 1 h at room temperature to form the immune-sandwich complexes. After washing with PBST buffer, 200 μL 1 μg/mL streptavidin was added to the beads, and incubated for 30 min at 37 °C. The beads were then washed with PBST buffer, and the biotin-labeled trigger DNA (53 μL 1 μM) was added to incubate for another 30 min at 37 °C. After the biotin-streptavidin-biotin bridging step was completed, NHCR probes (Substrate A: Substrate B = 1:2) were added to the beads and incubated for 1 h at 37 °C. After three times washing, the beads were resuspended in 300 μL PBST and immediately detected by flow cytometry. Under the optimal conditions, the total analysis time of this method is 5 h. The high throughput of the flow cytometer enables the detection of a sample within 10 s. The mean fluorescence intensity (MFI) of 10,000 MBs for each sample was detected through the FL1 (FAM/FITC) channel under the 488 nm laser excitation.

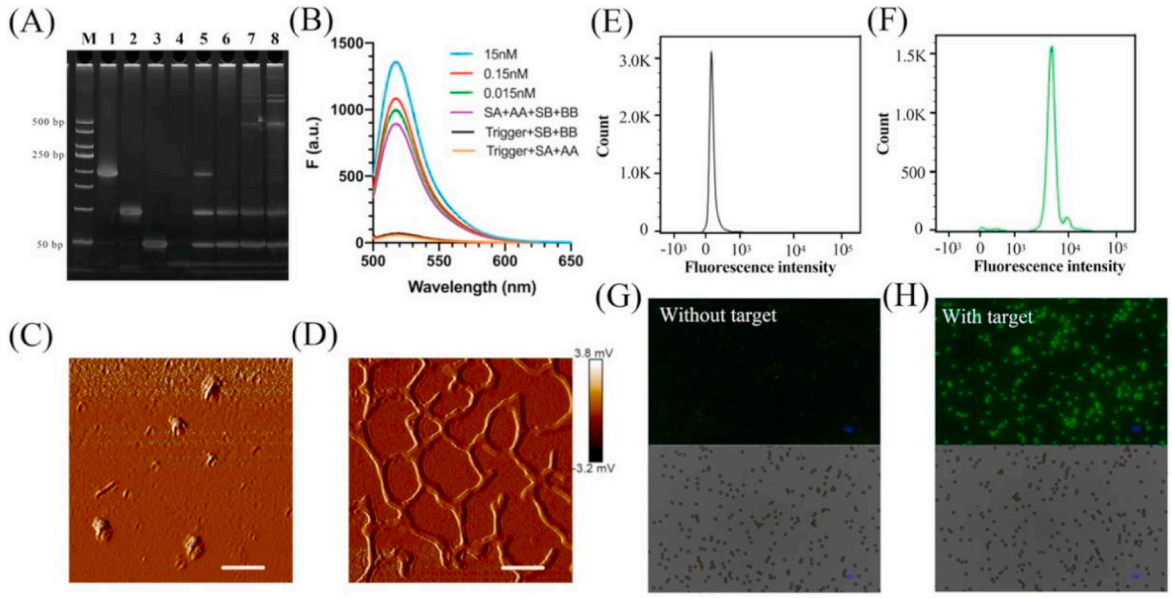


Fig. 1. Feasibility of immune-NHCR. (A) 15% PAGE gel electrophoresis of the NHCR self-assembly mechanism. Lane M: DNA marker; Lane 1: Substrate A; Lane 2: Substrate B; Lane 3: by-product A; Lane 4: by-product B; Lane 5: Substrate A, Substrate B, Assistant A, Assistant B (without target); Lane 6–8: nonlinear hybridization chain reaction system with 15, 75, 150 nM target DNA, respectively. (B) The fluorescent spectrum of different mixtures. The above three curves represent complete NHCR with different trigger concentrations. (Trigger + SA + AA + SB + AB; SA, AA, SB, AB represent Substrate A, Assistant A, Substrate B, Assistant B respectively). The fourth curve is the blank control without trigger, but retains the SA, AA, SB and AB. The blow two curves test the spontaneous reaction, the trigger concentration in these two curves is 0.15 nM. (C, D) AFM imaging of the assembled products of NHCR in the presence of 0 nM (C) and 15 nM trigger(D). Scale bar: 200 nm. (E) Flow cytometry results of MBs in the absence of target protein. (F) Flow cytometry results of MBs in the presence of 1 ng/mL PSA. (G) Inverted fluorescence microscope results of MBs in the absence of target protein. (H) Inverted fluorescence microscope results of MBs in the presence of 1 ng/mL PSA.

1.6. AFM imaging

The amplified NHCR product was diluted with NHCR Buffer, then 10 μL of the product was deposited on a clean mica sheet surface, and left for about 3 min. The mica sheet was subsequently rinsed with ultrapure water three times, and dried with nitrogen. AFM experiment was then conducted by AFM Bruker Multimode 8.

1.7. Gel electrophoresis

To verify the amplification process, 15% native polyacrylamide gel electrophoresis (PAGE) was carried out on ice under a constant potential of 100 V for 120 min. After being stained by Gel-Red for 10 min, the gel was scanned with ChemiDoc XRS + molecular imager.

1.8. Detection of PSA in the blood sample

The patient's blood samples were obtained from the Third Xiangya Hospital (Changsha, China). After centrifugation at 4000 rpm for 10 min, the serum was separated from the whole blood and collected at −20 °C for further use. The PSA level in blood samples was tested according to the above-mentioned immune-NHCR analytical process with different diluted ratios (We will first dilute the sample by a factor of 10, and if the detected value exceeds the threshold, we will continue to dilute it down by a factor of 100, and so on.). Samples 1, 4, and 5 were diluted 100 times; sample 2 was diluted 200 times and all samples were added at 1000 μL. Finally, the magnetic beads that completed the whole immune-NHCR analysis were re-suspended in 300 μL PBST buffer and quantified by flow cytometry. Additionally, the immune-NHCR assay results were compared with those reported by the hospital using the chemiluminescence immunoassay.

2. Results and discussion

2.1. Immunoassay and detection principle

The principle of this immune-NHCR strategy for the detection of PSA was schematically shown in Scheme 1. The method consisted of three cascade steps: the specific recognition of antigen and antibodies; streptavidin involved bridging step between biotin-labeled detection antibody and biotin-labeled trigger DNA, and NHCR amplification step. During the detection process, the surface of the carboxylated MBs was modified with capture antibody, which was able to capture the PSA antigen specifically. Biotin-labeled detection antibodies were then added to form a double-antibody sandwich complex on the MBs. Afterward, streptavidin and biotin-labeled trigger DNA was introduced to the MBs *via* the biotin-avidin system. Each trigger DNA could initiate the autonomous self-assembly of DNA dendrimer *via* two double strand probes (Substrate A and Substrate B), and two single strand probes (Assistant A and Assistant B). The substrate A and B were designed with 1–2 loop structures in the middle and toehold structure at one end, which were formed by annealing two partially complementary single strands: F chain (modified with FAM) and Q chain (modified with BHQ-1). Assistant A and B strands were designed to have completely complementary domains for their Q chains respectively. Due to the close proximity of the fluorophore and quencher in the formed Substrate A/B, fluorescence on the Substrate A/B was quenched. Upon introduction of trigger DNA, it hybridized with the toehold region on the Substrate A and thus opened the first bulge loop. Subsequently, Assistant A invaded Substrate A *via* branch migration reaction, replacing the Q chain from its original structure with the generation of byproduct A. At this point, the two loops were opened completely and thus exposed two identical toehold sequences, which consecutively hybridized with Substrate B. Assistant B displaced the Q chain in the same manner to form byproduct B, and the exposed new toehold region on the F chain was regarded as a new trigger which was ready to initiate new rounds of strand displacement reaction.

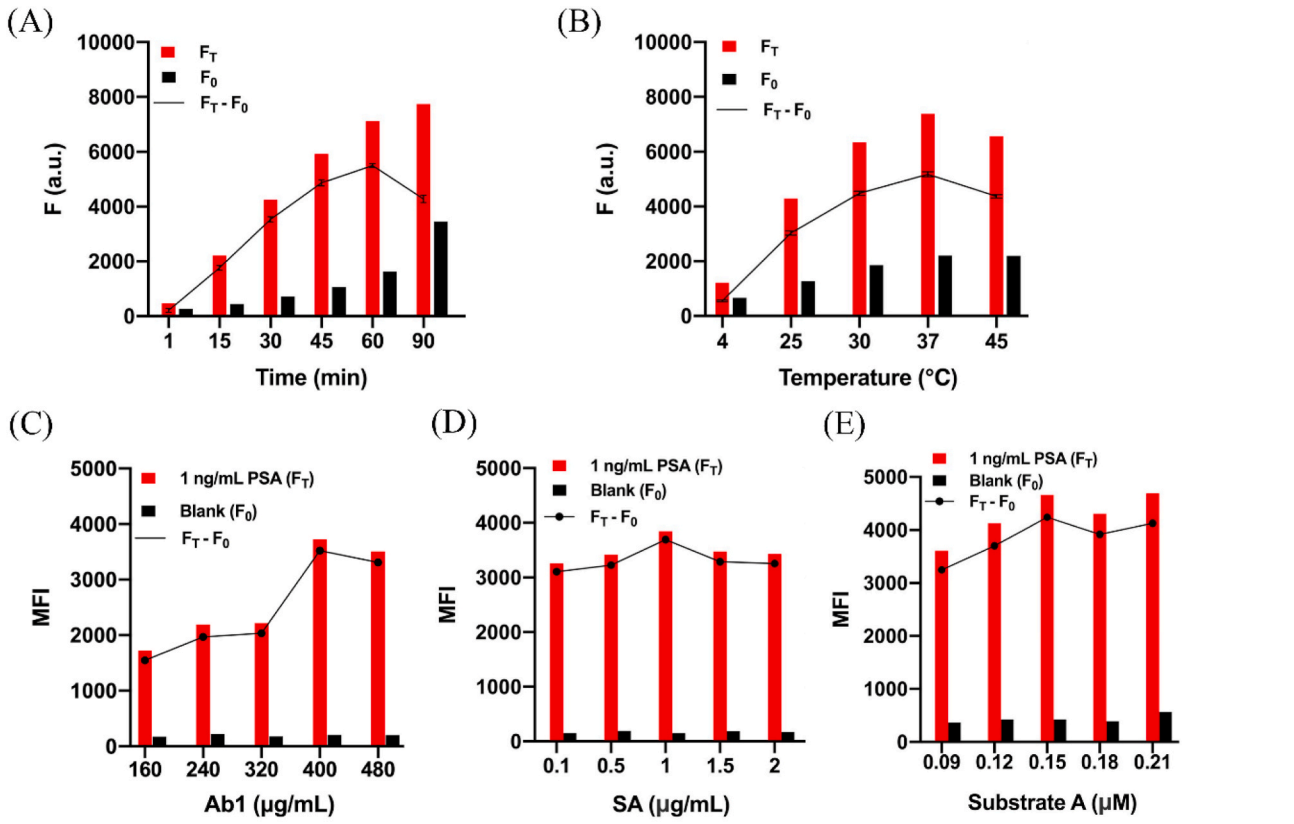


Fig. 2. Optimization of experiment parameter on the fluorescence signals of the immune-NHCR strategy. (A) The reaction time and (B) temperature of NHCR amplification process are optimized. F_T represents trigger DNA concentration of 15 nM; F_0 represents in the absence of trigger DNA. F_T - F_0 represents the fluorescence difference between F_T and F_0. Error bars: SD; n = 3. (C) The concentration of Ab1, (D) the concentration of streptavidin, (E) the concentration of Substrate A of immuno-NHCR process are optimized.

The chain-branching growth of DNA dendrimer caused the fluorophore on the F chain to be continuously turned on, producing an exponentially increasing fluorescent signal on the MBs. The quantitative detection of target proteins was achieved by flow cytometry detection of the average fluorescence intensity on the MBs. Statistical analysis of the fluorescence signal collected from each sample by flow cytometry could accurately quantify the target PSA level in terms of mean fluorescence intensity (MFI) with low detection of limit and high selectivity.

2.2. Feasibility of NHCR nanoassembly

As the signal output strategy of the immunosensor, validation of NHCR is crucial for the performance and accuracy of the results. Native polyacrylamide gel electrophoresis (PAGE) was utilized to verify the self-assembly of DNA dendrimers. As illustrated in Fig. 1A, bands in lane 2, lane 3, lane 4, and lane 5 were on behalf of Substrate A, Substrate B, by-product A, and by-product B, separately. In the absence of target DNA, the NHCR was unable to be initiated and the high molecule weight dendrimer was invisible (lane 5). However, upon the introduction of trigger DNA, series of bright bands with lower mobility appeared, indicating the assembly of dendrimer (lane 6–8). Moreover, the brightness of the bands with lower mobility increased with the increase of trigger concentration, which suggested the potential for quantitative analysis. As shown in Fig. 1B, the mixture of Trigger, Substrate A and Assistant A; the mixture of Trigger, Substrate B and Assistant B had a relatively weak fluorescent signal which indicated that the two substrate strands were quite stable in the buffer solution. Conversely, a significant increase in fluorescence intensity occurred when the trigger DNA was introduced to the integrity NHCR system (Trigger + SA + AA + SB + BB). With the increase of trigger concentration, the corresponding fluorescence value also gradually increased, which proved the feasibility of this amplification method.

In order to further confirm the PAGE results and reveal that the assembled products of NHCR are the expected dendritic morphology, we used atomic force microscopy (AFM) to image the assembled product directly. As shown in Fig. 1C and D, when there is no DNA trigger, AFM images only showed some tiny spots. These spots represented unassembled substrates, assistants and preformed by-products, meaning that there was no NHCR reaction. In the presence of DNA trigger, large-scale dendrimers nanostructures were observed in AFM images, showing that the NHCR reaction happened as expected.

2.3. Feasibility of immune-NHCR strategy

To evaluate the sensing performance of the proposed immune-NHCR strategy, the feasibility of the above methodology was testified by fluorescence microscope observation and flow cytometry analysis. Fig. 1E and F are the fluorescence histogram of MBs of the control group without adding target PSA and the test group with target PSA measured by flow cytometry, respectively. We can see that the fluorescence intensity of the MBs of the control group was weak, which was obviously different from the test group. The difference of fluorescence intensity between the MBs with target PSA and the MBs without target showed that the immune-NHCR strategy was feasible. In order to see the difference of the fluorescence intensity between the test group and the control group more intuitively, we observed the fluorescence intensity of the MBs of the control group without target PSA and the test group with 1 ng/mL PSA under the inverted fluorescence microscope. As shown in Fig. 1G and H, in the absence of target PSA, there was almost no fluorescence on the MBs. The fluorescence dendrimer could not be formed on the MBs without target, and any subsequent addition of reactants was removed by magnetic separation, resulting in almost no

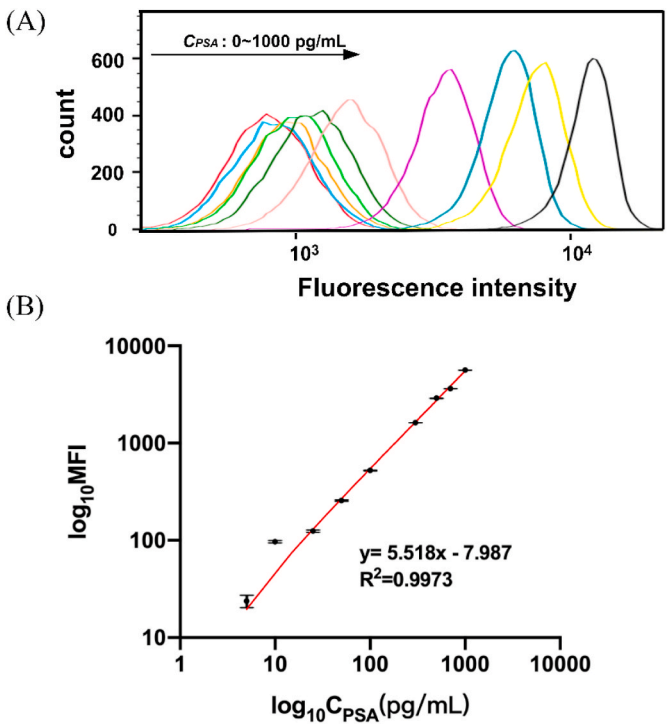


Fig. 3. (A) Histograms of fluorescence response of the immune-NHCR assay in the presence of different concentrations of PSA. (B) The corresponding calibration plot between the logarithm of MFI and the logarithm of PSA concentration (0, 5, 10, 25, 50, 100, 300, 500, 700, 1000 pg/mL). The error bars represent the standard deviation of four replicates for each point.

background signal. Conversely, a significant increase in the fluorescence of MBs was observed when the target antigen appeared in the system. This means that a large amount of fluorescence dendrimer was produced on the MBs. It also proved that the sandwich-type immune-NHCR strategy was feasible.

2.4. Optimization of the immunosensor

To achieve highly sensitive sensing performance, various experimental parameters need to be optimized (Fig. 2). In terms of NHCR process, the reaction time is crucial for the efficiency of the immunosensor. As shown in Fig. 2A, beyond 60 min, the signal of fluorescence achieved a plateau. Therefore, the reaction time was optimized to be 60 min. Moreover, the reaction temperature has a great influence on the stability and kinetics of the NHCR process. The temperature was set up ranging from 4 to 45 °C to apply to the dendrimer assembly process. As shown in Fig. 2B, the signal rose to a maximum value at 37 °C and decreased gradually with the increase of temperature. Hence, we selected 37 °C as the optimized NHCR reaction temperature.

In terms of the immunoassay process, the appropriate amount of capture antibody is very important to the performance of the sensor, because the amount of antibody affects the formation of MB-Ab1 complex and the ability of the MBs to capture target protein. Fig. 2C showed that the fluorescence signal stabilized when the amount of Ab1 reached 400 μg/mL. Therefore, the antibody level of 400 μg/mL was chosen for the follow-up experiments. Streptavidin plays an important role in immunosensors, as it acts as a bridge to link the double antibody sandwich complex and the NHCR amplification system. The appropriate amount of streptavidin determines the performance of the assay. As displayed in Fig. 2D, the fluorescence signal was enhanced when the concentration of streptavidin changed from 0.1 to 2 μg/mL, and then gradually descend. Accordingly, 1 μg/mL streptavidin was adopted in our experiments. The concentration of substrate A and B also influences

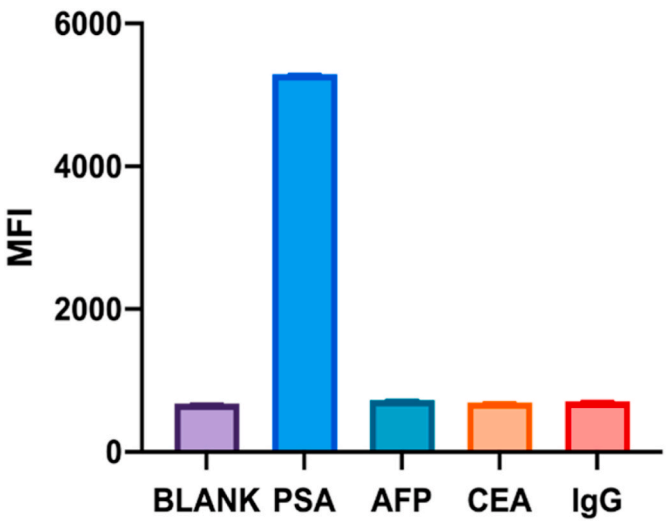


Fig. 4. Selectivity of this demonstrated method for different biomarkers: PSA (1 ng/mL), AFP (500 ng/mL), CEA (500 ng/mL), and IgG (500 ng/mL). The error bars represent the standard deviation of three replicates for each point.

the performance of the immunosensor, since high concentrations may cause an increase in the background and low concentrations reduce the dendrimer forming process, resulting in long reaction times. Therefore, as shown in Fig. 2E, the fluorescence intensity increased with the increment of Substrate A, and the signal tended to be steady subsequently. Hence, 0.15 μM was chosen as the optimized concentration of substrate A for further use.

2.5. Analytical performance

In order to verify the analytical performance of the immune-NHCR strategy, quantitative detection of PSA was performed under the above optimal parameters. As shown in Fig. 3A, the fluorescence histograms recorded from the flow cytometer illustrated that the mean fluorescent intensity (MFI) increased according to the increase of PSA concentration from 0 pg/mL to 1000 pg/mL. Moreover, Fig. 3B revealed a good dynamic linear range between the logarithm of MFI and the logarithm of PSA concentration in the range from 0 pg/mL to 1000 pg/mL. The linear regression equation was $y = 5.518\ x - 7.987$ ($R^2 = 0.9973$, where y and x represented the logarithm of MFI signal and the logarithm of PSA concentration, respectively), and the detection limit (LOD) was 1.92 pg/mL which was comparable to previously reported method for PSA detection (Table S1). The immune-NHCR strategy utilized the self-assembly amplification characteristics of the nonlinear hybridization chain reaction, and selected MBs to immobilize and enrich the targets protein, which avoided non-specific adsorption through magnetic separation, greatly reducing the background signal and providing relatively low detection of limit.

Furthermore, to evaluate the selectivity of the biosensor, several common interfering proteins were added to perform the immune-NHCR procedure, including AFP, CEA and IgG. In this experiment, the concentration of other proteins was 500 ng/mL, while the PSA was only 1 ng/mL. As shown in Fig. 4, even though the input concentration was 500-fold higher than that of PSA, the signal produced by the interfering protein had ignorable difference with the blank group, indicating the excellent selectivity of our immunoassay, which enabled the accurate detection of target proteins in complex biological samples.

2.6. Detection of PSA in clinical serum sample

To further investigate the clinical feasibility and practicability of this immunosensor, we extracted serums from the blood samples of three

Table 1
Detection of PSA in the real serum samples.

Sample	Immune-NHCR (ng/mL)	Dilution ratio	Signal (a.u.)	RSD (%)	Date from hospital (ng/mL)	Dilution ratio	Signal (RLU)	RSD(%)
1	2.19 ± 0.02	100x	965 ± 8.33	1.11	2.03 ± 0.1	1x	14727.18 ± 101	4.94
2	87.93 ± 0.80	200x	1579 ± 13.75	0.91	85.08 ± 6.19	1x	4786356.21 ± 2173191.39	7.28
3	0.139 ± 0.01	1x	742 ± 35	7.37	0.12 ± 0.01	1x	12921.66 ± 10.50	9.80
4	14.05 ± 0.32	100x	744 ± 11.02	2.29	13.82 ± 1.45	1x	33192.35 ± 3381.03	10.52
5	58.52 ± 1.68	100x	1985 ± 57.45	2.87	56.30 ± 2.07	1x	612299.78 ± 86819.67	3.68

*Sample 1, 4, 5 were diluted 100 times; sample 2 were diluted 200 times, the input volume of each sample is 1000 μL. RLU: Relative light Units.

patients and detected their corresponding PSA levels using the immune-NHCR strategy. Meanwhile, the samples were also measured by the Third Xiangya hospital (Changsha 410013, China) using chemiluminescence immunoassay. The results shown in Table 1 indicated that this NHCR amplification-based fluorescence immunoassay showed great promise in the accurate detection of biomarkers in complex sample matrix. This immune-NHCR based immunoassay provide advantages over conventional PSA testing methods (Table 1S), such as lower detection of limit, stability signal output, enzyme-free procedures, and high throughput analysis. Compared to the hospital method, the concentration we measured was proportional to the signal. Whereas the concentration from hospital does not appear to be, but is proportional to the logarithmic value of the signal. Notably, the results of the diluted serum samples are still consistent with the undiluted hospital data and had smaller RSD (%) values, indicating our method is more robust and have the potential for detecting lower concentrations samples. In other words, our method can not only detect significantly elevated PSA levels in prostate cancer patients, but we can also use it to monitor slight fluctuations in PSA levels in patients after clinical treatment, and can even be used to detect trace amounts of biomarkers by changing antibody pairs.

3. Conclusion

In summary, a novel NHCR based immunoassay with low detection limit, high selectivity, low background was developed for prostate-specific antigen biomarkers detection. Under the best experimental conditions, our method can detect PSA with a concentration as low as 1.92 pg/mL, and show much higher selectivity to target PSA than non-specific antigen with a concentration 500 times that of PSA. In practical application, the PSA content of several prostate cancer patients detected by this method is very close to the results measured by the Third Xiangya Hospital, meaning that this method is stable and reliable. In a word, this method has the potential to be applied to medical research and clinical detection.

This research method has many other potential advantages besides the ones mentioned above. On the one hand, we can only replace the antibody pairs on MBs instead of the whole system to realize the detection of other protein antigens. On the other hand, we use flow cytometry to report the results of this immunoassay, meaning that there will be some advantages compared with other methods like fluorescence spectrophotometer and microplate reader. Firstly, we can realize the simultaneous detection of different target antigens in flow analysis by capturing different antigens on magnetic beads with different sizes. We can distinguish the signals of different antigens according to the difference of particle sizes. Secondly, measuring the fluorescence intensity on magnetic beads instead of solution by flow cytometry can avoid the problem of inaccurate measurement caused by the deposition of magnetic beads in solution by other methods. Thirdly, flow cytometry can fix 10,000 magnetic beads and measure their fluorescence intensity every time to make the measurement more accurate, but other methods can't ensure that the number of magnetic beads measured each time is consistent. Lastly, compared with fluorescence spectrophotometer and microplate reader, flow cytometry has the advantages of high sensitivity and high throughput. Although the application of flow cytometry has not been popularized, which means that this method will have some limitations in practical application. But in the last five years, analysis research based on flow cytometry began to emerge in large numbers and has an increasing application trend [37–40]. We believe that with the advantages of flow cytometry and the progress of science and technology, flow cytometry will become as common and widely used as fluorescence photometer. Therefore, it is promising to use flow cytometry to report the signal of this method.

Generally speaking, the immunoassay developed by us has many potential advantages. Although our method takes a long time to analyze target protein, which is not conducive to the subsequent on-site inspection. We have done some work to reduce the analysis time and already have made great progress. We will report related work in the near future. We believe that when we solve this deficiency, this immunosensor based on NHCR will be widely used in clinical diagnosis and poct (point of care testing).

CRediT authorship contribution statement

Zhuoer Zeng: Conceptualization, Writing – original draft, Formal analysis. **Rong Zhou:** Conceptualization, Writing – original draft, Data curation, Formal analysis. **Ruowei Sun:** Resources. **Xun Zhang:** Resources. **Di Zhang:** Resources. **Qubo Zhu:** Writing – review & editing. **Chuanpin Chen:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2022.340048.

References

[1] S. Hou, T. Feng, N. Zhao, J. Zhang, H. Wang, N. Liang, L. Zhao, A carbon nanoparticle-peptide fluorescent sensor custom-made for simple and sensitive detection of trypsin, J. Pharmaceut. Anal. 10 (2020) 482–489, https://doi.org/10.1016/j.jpha.2020.08.009.
[2] S. Vigneshvar, C.C. Sudhakumari, B. Senthilkumaran, H. Prakash, Recent advances in biosensor technology for potential applications – an overview, Front. Bioeng. Biotechnol. 4 (2016) 11, https://doi.org/10.3389/fbioe.2016.00011.
[3] Y. Wang, Y. Wang, D. Wu, H. Ma, Y. Zhang, D. Fan, X. Pang, B. Du, Q. Wei, Label-free electrochemical immunosensor based on flower-like Ag/MoS2/rGO nanocomposites for ultrasensitive detection of carcinoembryonic antigen, Sensor.

Actuator. B Chem. 255 (2018) 125–132, https://doi.org/10.1016/j.snb.2017.07.129.
[4] R. Gao, Z. Cheng, A.J. deMello, J. Choo, Wash-free magnetic immunoassay of the PSA cancer marker using SERS and droplet microfluidics, Lab Chip 16 (2016) 1022–1029, https://doi.org/10.1039/C5LC01249J.
[5] Z. Gao, L. Hou, M. Xu, D. Tang, Enhanced colorimetric immunoassay accompanying with enzyme cascade amplification strategy for ultrasensitive detection of low-abundance protein, Sci. Rep. 4 (2015) 3966, https://doi.org/10.1038/srep03966.
[6] Y.-H. Cheng, H. Tang, R.-Q. Yu, J.-H. Jiang, DNA-Programmed plasmonic ELISA for the ultrasensitive detection of protein biomarkers, Analyst 145 (2020) 4860–4866, https://doi.org/10.1039/D0AN00656D.
[7] S.R. Yousefi, A. Sobhani, M. Salavati-Niasari, A new nanocomposite superionic system (CdHgI4/HgI2): synthesis, characterization and experimental investigation, Adv. Powder Technol. 28 (2017) 1258–1262, https://doi.org/10.1016/j.apt.2017.02.013.
[8] S.R. Yousefi, O. Amiri, M. Salavati-Niasari, Control sonochemical parameter to prepare pure Zn0.35Fe2.65O4 nanostructures and study their photocatalytic activity, Ultrason. Sonochem. 58 (2019), 104619, https://doi.org/10.1016/j.ultsonch.2019.104619.
[9] S.R. Yousefi, M. Ghanbari, O. Amiri, Z. Marzhoseyni, P. Mehdizadeh, M. Hajizadeh-Oghaz, M. Salavati-Niasari, Dy $_2$ BaCuO $_5$/Ba $_4$ DyCu $_3$ O $_{9.09}$ S-scheme heterojunction nanocomposite with enhanced photocatalytic and antibacterial activities, J. Am. Ceram. Soc. 104 (2021) 2952–2965, https://doi.org/10.1111/jace.17696.
[10] S.R. Yousefi, A. Sobhani, H.A. Alshamsi, M. Salavati-Niasari, Green sonochemical synthesis of BaDy $_2$ NiO $_5$/Dy $_2$ O $_3$ and BaDy $_2$ NiO $_5$/NiO nanocomposites in the presence of core almond as a capping agent and their application as photocatalysts for the removal of organic dyes in water, RSC Adv. 11 (2021) 11500–11512, https://doi.org/10.1039/D0RA10288A.
[11] S.R. Yousefi, M. Masjedi-Arani, M.S. Morassaei, M. Salavati-Niasari, H. Moayedi, Hydrothermal synthesis of DyMn2O5/Ba3Mn2O8 nanocomposite as a potential hydrogen storage material, Int. J. Hydrogen Energy 44 (2019) 24005–24016, https://doi.org/10.1016/j.ijhydene.2019.07.113.
[12] S.R. Yousefi, D. Ghanbari, M. Salavati-Niasari, M. Hassanpour, Photo-degradation of organic dyes: simple chemical synthesis of Ni(OH)2 nanoparticles, Ni/Ni(OH)2 and Ni/NiO magnetic nanocomposites, J. Mater. Sci. Mater. Electron. 27 (2016) 1244–1253, https://doi.org/10.1007/s10854-015-3882-6.
[13] S.R. Yousefi, H.A. Alshamsi, O. Amiri, M. Salavati-Niasari, Synthesis, characterization and application of Co/Co3O4 nanocomposites as an effective photocatalyst for discoloration of organic dye contaminants in wastewater and antibacterial properties, J. Mol. Liq. 337 (2021), 116405, https://doi.org/10.1016/j.molliq.2021.116405.
[14] L. Chang, J. Li, L. Wang, Immuno-PCR: an ultrasensitive immunoassay for biomolecular detection, Anal. Chim. Acta 910 (2016) 12–24, https://doi.org/10.1016/j.aca.2015.12.039.
[15] W. Witkowska McConnell, C. Davis, S.R. Sabir, A. Garrett, A. Bradley-Stewart, P. Jajesniak, J. Reboud, G. Xu, Z. Yang, R. Gunson, E.C. Thomson, J.M. Cooper, Paper microfluidic implementation of loop mediated isothermal amplification for early diagnosis of hepatitis C virus, Nat. Commun. 12 (2021) 6994, https://doi.org/10.1038/s41467-021-27076-z.
[16] H. Qi, S. Yue, S. Bi, C. Ding, W. Song, Isothermal exponential amplification techniques: from basic principles to applications in electrochemical biosensors, Biosens. Bioelectron. 110 (2018) 207–217, https://doi.org/10.1016/j.bios.2018.03.065.
[17] R.M. Dirks, N.A. Pierce, Triggered amplification by hybridization chain reaction, Proc. Natl. Acad. Sci. Unit. States Am. 101 (2004) 15275–15278, https://doi.org/10.1073/pnas.0407024101.
[18] S. Lu, T. Hu, S. Wang, J. Sun, X. Yang, Ultra-sensitive colorimetric assay system based on the hybridization chain reaction-triggered enzyme cascade amplification, ACS Appl. Mater. Interfaces 9 (2017) 167–175, https://doi.org/10.1021/acsami.6b13201.
[19] Z. Ge, M. Lin, P. Wang, H. Pei, J. Yan, J. Shi, Q. Huang, D. He, C. Fan, X. Zuo, Hybridization chain reaction amplification of MicroRNA detection with a tetrahedral DNA nanostructure-based electrochemical biosensor, Anal. Chem. 86 (2014) 2124–2130, https://doi.org/10.1021/ac4037262.
[20] Y. Zhang, Z. Chen, Y. Tao, Z. Wang, J. Ren, X. Qu, Hybridization chain reaction engineered dsDNA for Cu metallization: an enzyme-free platform for amplified detection of cancer cells and microRNAs, Chem. Commun. 51 (2015) 11496–11499, https://doi.org/10.1039/C5CC03144C.
[21] Z. Li, X. Miao, Z. Cheng, P. Wang, Hybridization chain reaction coupled with the fluorescence quenching of gold nanoparticles for sensitive cancer protein detection, Sensor. Actuator. B Chem. 243 (2017) 731–737, https://doi.org/10.1016/j.snb.2016.12.047.
[22] R. Zhou, Z. Zeng, R. Sun, W. Liu, Q. Zhu, X. Zhang, C. Chen, Traditional and new applications of the HCR in biosensing and biomedicine, Analyst 146 (2021) 7087–7103, https://doi.org/10.1039/D1AN01371H.
[23] G. Xu, M. Lai, R. Wilson, A. Glidle, J. Reboud, J.M. Cooper, Branched hybridization chain reaction—using highly dimensional DNA nanostructures for label-free, reagent-less, multiplexed molecular diagnostics, Microsyst. Nanoeng. 5 (2019) 37, https://doi.org/10.1038/s41378-019-0076-z.
[24] J. Dong, Z. Zeng, R. Sun, X. Zhang, Z. Cheng, C. Chen, Q. Zhu, Specific and sensitive detection of CircRNA based on netlike hybridization chain reaction, Biosens. Bioelectron. 192 (2021), 113508, https://doi.org/10.1016/j.bios.2021.113508.
[25] S. Li, P. Li, M. Ge, H. Wang, Y. Cheng, G. Li, Q. Huang, H. He, C. Cao, D. Lin, L. Yang, Elucidation of leak-resistance DNA hybridization chain reaction with universality and extensibility, Nucleic Acids Res. 48 (2020) 2220–2231, https://doi.org/10.1093/nar/gkaa016.
[26] Z. Zeng, R. Zhou, R. Sun, X. Zhang, Z. Cheng, C. Chen, Q. Zhu, Nonlinear hybridization chain reaction-based functional DNA nanostructure assembly for biosensing, bioimaging applications, Biosens. Bioelectron. 173 (2021), 112814, https://doi.org/10.1016/j.bios.2020.112814.
[27] F. Xuan, I.-M. Hsing, Triggering hairpin-free chain-branching growth of fluorescent DNA dendrimers for nonlinear hybridization chain reaction, J. Am. Chem. Soc. 136 (2014) 9810–9813, https://doi.org/10.1021/ja502904s.
[28] X. Ding, Y. Wang, W. Cheng, F. Mo, Y. Sang, L. Xu, S. Ding, Aptamer based electrochemical adenosine triphosphate assay based on a target-induced dendritic DNA nanoassembly, Microchim. Acta 184 (2017) 431–438, https://doi.org/10.1007/s00604-016-2026-x.
[29] C.-C. Chang, C.-Y. Chen, T.-L. Chuang, T.-H. Wu, S.-C. Wei, H. Liao, C.-W. Lin, Aptamer-based colorimetric detection of proteins using a branched DNA cascade amplification strategy and unmodified gold nanoparticles, Biosens. Bioelectron. 78 (2016) 200–205, https://doi.org/10.1016/j.bios.2015.11.051.
[30] X. Ding, W. Cheng, Y. Li, J. Wu, X. Li, Q. Cheng, S. Ding, An enzyme-free surface plasmon resonance biosensing strategy for detection of DNA and small molecule based on nonlinear hybridization chain reaction, Biosens. Bioelectron. 87 (2017) 345–351, https://doi.org/10.1016/j.bios.2016.08.077.
[31] Y. Zhao, Y. Feng, Y. Zhang, P. Xia, Z. Xiao, Z. Wang, H. Yan, Combining competitive sequestration with nonlinear hybridization chain reaction amplification: an ultra-specific and highly sensitive sensing strategy for single-nucleotide variants, Anal. Chim. Acta 1130 (2020) 107–116, https://doi.org/10.1016/j.aca.2020.07.022.
[32] T. Han, S. Wang, F. Sheng, S. Wang, T. Dai, X. Zhang, G. Wang, Target triggered ultrasensitive electrochemical polychlorinated biphenyl aptasensor based on DNA microcapsules and nonlinear hybridization chain reaction, Analyst 145 (2020) 3598–3604, https://doi.org/10.1039/D0AN00065E.
[33] Y. Xu, Y. Xu, Z. Zuo, X. Zhou, B. Guo, Y. Sang, S. Ding, Triggered hairpin switch and in situ nonlinear hybridization chain reaction enabling label-free electrochemiluminescent detection of BCR/ABL fusion gene, J. Electroanal. Chem. 801 (2017) 192–197, https://doi.org/10.1016/j.jelechem.2017.07.050.
[34] A. Fuerea, G. Baciarello, A. Patrikidou, L. Albigès, C. Massard, M. Di Palma, B. Escudier, K. Fizazi, Y. Loriot, Early PSA response is an independent prognostic factor in patients with metastatic castration-resistant prostate cancer treated with next-generation androgen pathway inhibitors, Eur. J. Cancer 61 (2016) 44–51, https://doi.org/10.1016/j.ejca.2016.03.070.
[35] D.M. Rissin, C.W. Kan, T.G. Campbell, S.C. Howes, D.R. Fournier, L. Song, T. Piech, P.P. Patel, L. Chang, A.J. Rivnak, E.P. Ferrell, J.D. Randall, G.K. Provuncher, D. R. Walt, D.C. Duffy, Single-molecule enzyme-linked immunosorbent assay detects serum proteins at subfemtomolar concentrations, Nat. Biotechnol. 28 (2010) 595–599, https://doi.org/10.1038/nbt.1641.
[36] C.S. Thaxton, R. Elghanian, A.D. Thomas, S.I. Stoeva, J.-S. Lee, N.D. Smith, A. J. Schaeffer, H. Klocker, W. Horninger, G. Bartsch, C.A. Mirkin, Nanoparticle-based bio-barcode assay redefines "undetectable" PSA and biochemical recurrence after radical prostatectomy, Proc. Natl. Acad. Sci. Unit. States Am. 106 (2009) 18437–18442, https://doi.org/10.1073/pnas.0904719106.
[37] L. Zhu, D. Chen, X. Lu, Y. Qi, P. He, C. Liu, Z. Li, An ultrasensitive flow cytometric immunoassay based on bead surface-initiated template-free DNA extension, Chem. Sci. 9 (2018) 6605–6613, https://doi.org/10.1039/C8SC02752H.
[38] P. Nyeng, G.V. Dela Cruz, H. Semb, Flow cytometry detection of surface and intracellular antigens in pancreas from a single mouse embryo, Star Protoc. 2 (2021), 100636, https://doi.org/10.1016/j.xpro.2021.100636.
[39] R. Shandilya, A.M. Sobolev, N. Bunkar, A. Bhargava, I.Y. Goryacheva, P.K. Mishra, Quantum dot nanoconjugates for immuno-detection of circulating cell-free miRNAs, Talanta 208 (2020), 120486, https://doi.org/10.1016/j.talanta.2019.120486.
[40] A.A. Vora, P.K. Mondala, C. Costello, A.R. MacLeod, L.A. Crews, Sensitive intranuclear flow cytometric quantification of IRF4 protein in multiple myeloma and normal human hematopoietic cells, Star Protoc. 2 (2021), 100565, https://doi.org/10.1016/j.xpro.2021.100565.