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Multiplexed chemiluminescence imaging assay of protein biomarkers using DNA microarray with proximity binding-induced hybridization chain reaction amplification

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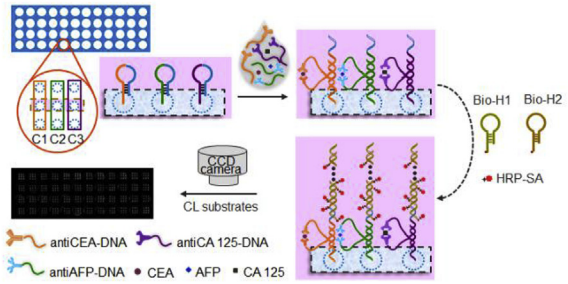
HIGHLIGHTS

- A chemiluminescence microarray imaging assay is developed for multiple proteins detection.
- The assay is performed on a DNA microarray with proximity binding-induced hybridization chain reaction.
- The assay is able to screen detect 3 protein biomarkers with good sensitivity and selectivity.
- This assay shows good extensibility by designing different spotting DNAs and antibody-DNA pairs.

GRAPHICAL ABSTRACT



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ABSTRACT

A multiplexed chemiluminescence (CL) imaging assay was designed for sensitive screen detection of a panel of protein biomarkers by integrating DNA microarray with proximity binding-induced hybridization chain reaction (HCR) amplification. The DNA microarray was manufactured by printing hairpin DNAs for different analytes detection in an array format onto the sensing cells arranged on an aldehyde-functionalized glass chip. The existence of target proteins induced the formation of sandwich immunocomplexes along with the hybridization of DNA strands labeled on the pair of antibodies via proximity effect, which subsequently unfolded their corresponding spotting hairpin DNAs to expose prelocked primer and trigger HCR assembly. Through the streptavidin-biotin conjugation, numerous horseradish peroxidase (HRP) was coupled on the HCR assemblies to catalyze H_2O_2-luminol reaction and produce amplified CL signals for sensitive CL imaging assay of protein targets. As a proof of concept, the DNA microarray was used to CL imaging assay of carcinoembryonic antigen, α-fetoprotein and carcinoma antigen 125 in ranges of 0.05–500 ng mL^{-1}, 0.06–600 ng mL^{-1} and 0.02–200 U mL^{-1}, respectively. The CL microarray imaging assay could also be extended to screen detect other biomarker groups by printing different hairpin DNAs on the microarray and using corresponding antibody-DNA pairs. The proposed CL microarray imaging assay showed advantages of small sample consumption, good cost effectiveness, and multiplex detection performance, exhibiting good applicability in clinical diagnosis.

1. Introduction

Cancer biomarkers are molecules secreted by tumor or specific responses of the body to the presence of cancers. The levels of

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cancer markers can indicate the occurrence and growth of cancers, for example, the assays of carcinoembryonic antigen (CEA) [1], α-fetoprotein (AFP) and carcinoma antigen 125 (CA 125) are accepted in clinical practice for monitoring colon, liver and ovarian cancers, respectively. However, most biomarkers are not specific to a particular tumor (e.g., CEA is associated with colorectal, liver, breast and pancreatic cancers), also most cancers have more than one biomarker associated with their incidence (e.g. Epithelial ovarian tumor is associated with CA 125, CEA, AFP and human chorionic gonadotropin (hCG)). Thus, accurate screen detection of a panel of cancer biomarkers is of extreme significance for early clinical diagnosis owning to the low disease specificity of single biomarker [2,3]. A variety of multiplex immunoassay methods have been developed for sensitive screen detection of protein biomarkers by using electrochemical [4–6], fluorescent [7,8] and chemiluminescent (CL) [9] readouts. Among them, array-based assays which can simultaneously detect different targets with a single label in different immunoreaction areas have gained most attentions [10–12]. Because there is no need of excitation light and complicated optical path, CL is an ideal readout of array-based immunoassays. However, due to the low CL signal from low quantum yields of CL reaction, the CL multiplex immunoassays are normally based on macro-protein arrays [13,14], which makes the detection throughput, sample and reagent consumption, and operation simplicity challenged.

Microarray which is defined as a collection of miniaturized spots arranged on a solid substrate permits many parallel measurements on the same specimen. Therefore, microarray-based multiplex immunoassay is capable to gain really high-throughput, low consumption, and good cost effectiveness. However, to date, only a few protein microarrays have been prepared for screen detection of cancer biomarkers [15,16] due to the preparation difficulty and low detection sensitivity [17]. In contrast, DNA microarrays can be prepared easier with lower cost, and are more stable and robust than protein microarrays because of the DNA characterizations of diversity, operability, easy synthesis and modification. In addition, by combining with powerful tool of nucleic acid amplifications, the DNA microarray normally shows sufficient detection sensitivity [18,19]. DNA microarrays are well-known for the preparation of protein microarrays [20], and fluorescent screen detection of DNA [21], microRNA [22] and bacteria [23], but few of them are reported for multiple detection of protein biomarkers.

The proximity binding-based immunoassay is a recently developed DNA-assisted protein assay technology in which the affinity recognition of proteins can be converted to DNA signal outputs [24]. These immunoassays use aptamers or antibody-DNA pairs to simultaneously recognize target protein, and the formation of sandwich immunocomplexes brings the tail sequences of aptamers or DNA pairs into close proximity with an increase of local concentration to allow subsequent DNA hybridizations [25,26]. Following the pioneering work of proximity ligation assay [27,28], other simple and sensitive proximity binding-based immunoassays have been developed with a variety of DNA assemblies [29], particularly with DNA-based signal amplification strategies, such as polymerase chain reaction [30], rolling circle amplification [31], exonuclease/endonuclease-mediated cycle amplification [32], hybridization chain reaction (HCR) [33], and catalytic hairpin assembly [34]. Because there is no need of thermal cycles and participation of enzymes, HCR is one of the most attractive isothermal amplification technique for biomolecules sensing [35,36]. Recently, HCR has also been employed to improve the detection performance of DNA microarray to obtain an fM level detect limit for fluorescent detection of DNA [18].

In this work, by integrating proximity binding-induced HCR with DNA microarray, a multiplexed CL imaging assay was designed for sensitive screen detection of CEA, AFP and CA 125. Here, DNA microarray was manufactured by spotting different functional hairpin DNAs onto the sensing cells arranged on an aldehyde-functionalized glass chip (Scheme 1). In the presence of target proteins, the sandwich immunocomplexes were formed to trigger the proximity hybridization of DNA strands labeled on the pair of antibodies and subsequently its reaction with spotting hairpin DNAs, resulting in the exposure of primer fragment to induce HCR along with the conjugation of horseradish peroxidase (HRP). The CL signal produced through the oxidation of luminol by H_2O_2 with HRP was collected by a charge-coupled device (CCD) for imaging assay of the protein targets. To the best of our knowledge, this was the first time using the DNA microarray to screen detect protein biomarkers, which avoided the requirement of complex and high cost protein microchips. In addition, by combining with HCR amplification, the sensitivity of the CL microarray was guaranteed. Although the method was applied to detect 3 protein biomarkers, it could be easily extended to screen detect 9 protein targets by designing different hairpin DNAs and antibody-DNA pairs. With the advantages of cost effectiveness, very small sample volume and multiple assay capability, the proposed CL microarray imaging assay held great promise for protein-related research and molecular disease diagnosis.

2. Materials and methods

2.1. Reagents

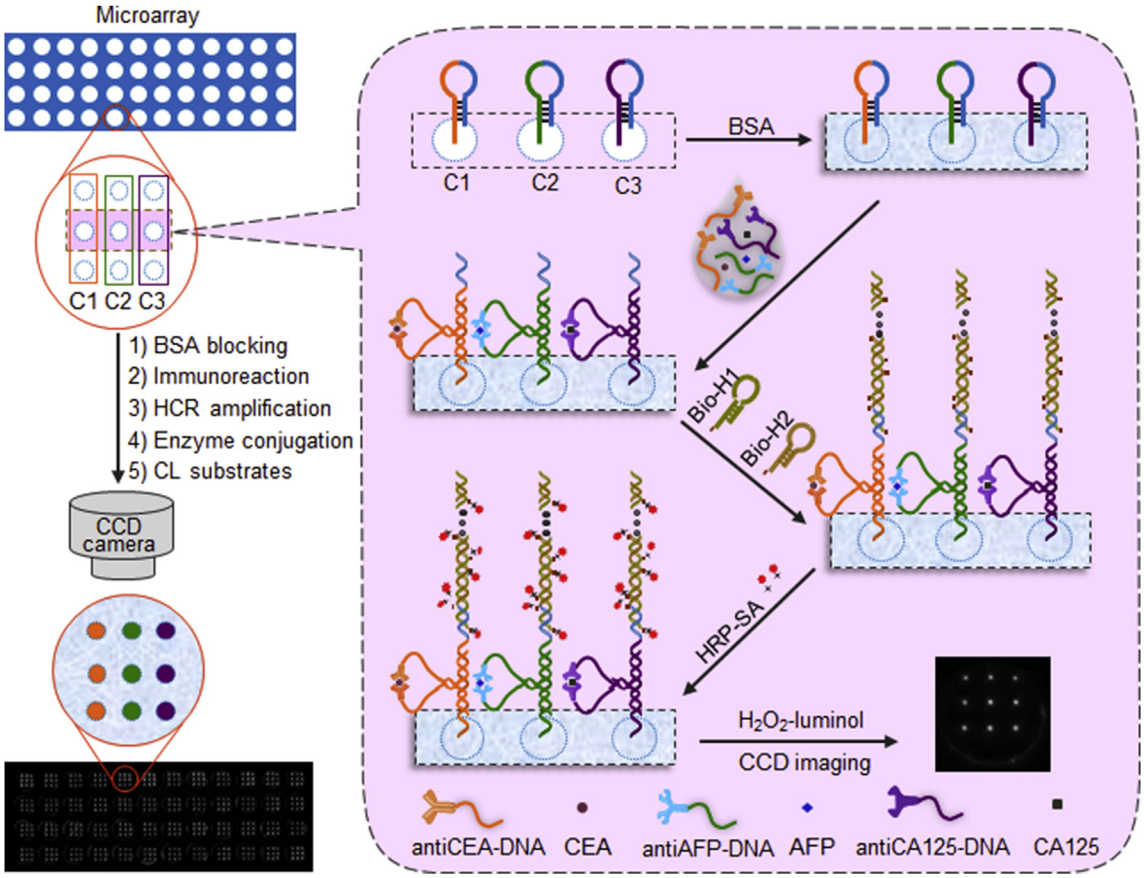
The oligonucleotides were synthesized by Shanghai Sangon Biotechnology Co. Ltd. (China) and their sequences were listed in Table S1 (supplementary). Antibodies of CEA (antiCEA, clone Nos. M58# and D3C#), AFP (antiAFP, clone Nos. 9K5# and 102K7#) and CA 125 (antiCA 125, clone Nos. 2H4# and 2D6#), as well as their standard antigen solutions were purchased from Beijing Keybiotech Co. Ltd. (China). CL substrate solution containing luminol-*p*-iodophenol and H_2O_2 was obtained from Autobio Diagnostics Co. Ltd. (China). HRP-Streptavidin (HRP-SA), tris(2-carboxyethyl) phosphine hydrochloride (TCEP), 3-maleimidobenzoic N-hydroxysuccinimide ester (MBS), tris(hydroxymethyl)aminomethane (Tris), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) was obtained from KeyGEN BioTECH Co. Ltd. (China). Ultrapure water obtained from a Millipore water purification system (≥18 MΩ, Milli-Q, Millipore) was used in all assays. All other reagents were of analytical grade and used without further purification. TE buffer (10 mM Tris-HCl, containing 140 mM NaCl and 5 mM $MgCl_2$, pH 7.4) was used as the stock and reaction buffer of oligonucleotides. PBS1 (10 mM, pH 5.5) and PBS2 (10 mM, pH 7.2) were used to prepare antibody-DNA. Washing buffer was PBS2 spiked with 0.01% Tween-20.

2.2. Apparatus

A cooled low-light CCD camera with high resolution (Bio-Spectrum 615 Imaging System, UVP, USA) was used to collect the CL images. A SmartArrayer 48 System (CapitalBio Corporation Co. Ltd., China) was used to print hairpin DNAs onto the sensing cells arranged on an aldehyde-functionalized glass chip.

2.3. Preparation of antibody-DNA

The antibody-DNA conjugates were prepared according to the reported procedure with some modifications [37]. Briefly, the antibodies (50 μg) were firstly reacted with a 40-fold excess of MBS in PBS2 with a total volume of 30 μL for 2 h at room temperature. Pure



Scheme 1. Schematic diagram of the multiplexed CL imaging assay using DNA microarray with proximity binding-induced HCR amplification.

antibody-MBS was obtained via ultrafiltration (50 kDa, 10000 rpm, 10 min, 8 times). Meanwhile, 30 μL of 100 μM thiolated oligonucleotide was reduced by a 100-fold molar excess of TCEP in PBS1 for 2 h and TCEP was removed by ultrafiltration (3 kDa, 10000 rpm, 10 min, 4 times). Then the purified antibody-MBS was mixed with their corresponding reduced thiol-DNAs for another incubation of 2 h at room temperature. Finally, the antibody-DNA conjugates were purified by ultrafiltration (50 kDa, 10000 rpm, 10 min, 8 times) and their concentrations were calibrated through a BCA protein assay kit (Thermo Scientific, USA). The antibody-DNA conjugates were stored at a concentration of 2 μM in PBS2 and were diluted to 100 nM with PBS2 before use. The successful preparation of antibody-DNA conjugates was confirmed by native polyacrylamide gel electrophoresis analysis and protein-staining methods (Fig. S1, supplementary).

2.4. Fabrication of DNA microarray

The DNA microarray was manufactured by printing hairpin DNAs (C1, C2 and C3) for different analytes detection in a 3 × 3 array format onto the sensing cells which were arranged in a 4 × 12 format on an aldehyde-functionalized glass chip (Scheme 1). Briefly, a hydrophobic photoinactive film was firstly pasted on the aldehyde-functionalized glass chip (Shanghai Baio Technology Co. Ltd., China) to construct 48 sensing cells (4 mm diameter) in a 4 rows × 12 columns format. Then, three amino modified hairpin DNAs (C1, C2 and C3) designed for CEA, AFP and CA 125 detection were printed on each sensing cells in a 3 rows × 3 columns format using the SmartArrayer with a spot diameter of 150 μm. The arrayed slides were incubated overnight at room temperature in a humidity environment. After being washed thoroughly with washing buffer, the sensing cells were immersed in 1% BSA for 30 min at 37 °C to block unreacted aldehyde groups. After washing, the DNA microarray was obtained and stored at 4 °C before use.

2.5. Detection of DNAs

Three pairs of DNAs (U1&D1, U2&D2, and U3&D3) were designed as targets to characterize the detection performance of the proposed CL microarray imaging assay. 6 μL of the mixture of these 6 DNA sequences at different concentrations in TE buffer was added to each sensing cell and incubated for 90 min. After washing and drying, 6 μL of 0.5 μM biotinylated HCR hairpins (Bio-H1 and Bio-H2) was placed on the sensing cell and incubated for 90 min. After another washing and drying, 6 μL of 10-fold diluted HRP-SA was delivered to the sensing cell and incubated for 45 min, followed by a washing and drying step. All the above reactions were carried out at 37 °C in a humidity chamber. Finally, 6 μL of CL substrate was added to the sensing cell and the CL image was recorded by CCD with a 3 min on chip model. The CL intensity of each microdot was identified by VisionWorksLS acquisition and analysis software (UVP) and calculated as the mean pixel intensity with consistent square around each microdot center.

2.6. Detection of protein biomarkers

1.2 μL of sample containing target proteins at different concentrations or serum sample from cancer patients (supplied by Jiangsu Cancer Hospital) was mixed with 4.8 μL of affinity solution containing antibody-DNA pairs at 50 nM and then dropped on the

sensing cell for incubation of 90 min at 37 °C in a humidity chamber. Followed by the same incubation steps in DNA detection with HCR probes and HRP-SA, the CL substrate was added for CL imaging. The CL intensity of microdots was identified and calculated as the same way described in DNA detection.

3. Results and discussion

3.1. Feasibility of the CL microarray imaging assay

In this work, the multiplexed CL imaging assay was designed on a DNA microarray with proximity binding-induced HCR amplification for sensitive screen detection of protein biomarkers (Scheme 1). Amino modified hairpin DNAs (C1, C2 and C3) containing a functional fragment of 20 bases, a stem of 24 bases and a loop of 18 bases were spotted on the sensing cells on aldehyde-functionalized glass chip for CEA, AFP and CA 125 detections. The initiate primer of HCR was prelocked in the hairpin structure in C1, C2 and C3, and could be exposed to trigger HCR after opening the hairpin DNAs to straight chains. Antibody-DNA pairs containing 7 complementary bases between DNA pairs (U'&D′-DNA) were designed to recognize target and subsequently open hairpin DNAs via proximity effect (Fig. S2, supplementary). Here, U1'&D1′, U2'&D2′, and U3'&D3′ DNA pairs had only 18&20, 17&18, and 17&18 complementary bases to C1, C2 and C3, respectively, thus, all of them were unable to open hairpin DNAs directly. However, in the presence of target proteins, the sandwich immunocomplexes were formed through the simultaneous binding of antibody pairs to target proteins, resulting in the close proximity of the DNA pairs with their local concentration increased dramatically to allow their hybridization with each other to produce long sequences with 38 or 35 bases complementary with C1, C2 or C3. These long sequences could open the spotted hairpin DNAs to expose prelocked primer for subsequent HCR assembly and HRP conjugation. Polyacrylamide gel electrophoresis analysis verified the HCR assembly of H1 and H2 in the presence of initiate sequence (I) (Fig. S3, supplementary). Benefiting from the HCR assembly, numerous HRP were eventually loaded on microdots for signal amplification and sensitive CL imaging assay of target proteins.

To verify the hybridization processes, three DNA pairs (U1&D1, U2&D2, and U3&D3) were used to mimic the sandwich immunocomplexes to open spotted hairpin DNAs and the subsequent HCR (Fig. 1). Here, U1&D1, U2&D2, and U3&D3 DNA pairs were designed to contain the same complementary bases to C1, C2 and C3 corresponding to U1'&D1′, U2'&D2′, and U3'&D3′ DNA pairs, respectively. But U1, U2 and U3 contained 16 bases in tail complementary with D1, D2 and D3, respectively, thus, the U1&D1, U2&D2, and U3&D3 DNA pairs were existed as T-shape DNA complexes with long sequences of 38 or 35 bases complementary with C1, C2 or C3, same as that in proximity immunocomplexes. As expected, neither U-DNA nor D-DNA lonely could open hairpin DNAs and produce CL signals (Fig. 1B and C). In contrary, obvious CL spots with strong CL intensities were observed on sensing cells with the co-existence of U&D DNA pairs. These results indicated only the assembly of DNA pairs through hybridization or proximity binding could produce long complementary sequences to open the spotted hairpin DNAs and expose primer for subsequent HCR amplification.

3.2. Optimization of detection conditions

Several key factors which affected the detection performance of the CL microarray imaging assay were investigated (Fig. 2). The density of hairpin DNAs (C1, C2 and C3) spotted on the glass slide was firstly studied by controlling the printing concentration of hairpin DNAs (Fig. 2A). Using C1 as an example, the assay showed a high CL signal as well as a high noise at high printing concentration. Although both CL signal and noise were decreased by decreasing the printing concentration, the signal-to-noise ratio increased. Concerning both the signal and signal-to-noise ratio, 0.5 μM of hairpin DNAs was used for the fabrication of DNA microarray.

To gain the best performance of HCR amplification, the concentration of HCR probes (H1 and H2) was also optimized. As shown in Fig. 2B, the CL intensity increased with increasing the concentration of H1 and H2 and reached the maximum value at 0.5 μM, then the CL intensity decreased. This might be because the long HCR assembly formed with high concentration of H1 and H2 produced steric hindrance to inhibit the conjugation of HRP-SA. In contrary, the noise was slightly changed with increasing the concentration of H1 and H2. Thus, a concentration of 0.5 μM was selected for H1 and H2 in this work.

3.3. Verification of HCR amplification

In order to evaluate the feasibility of HCR amplification, CL microarray imaging assays which used single DNA strand (H1) and HCR assembly to load HRP and produce CL signal were performed with the same detection conditions. Compared with the sensing cell incubated with H1, the sensing cell incubated with the mixture of H1 and H2 showed brighter dots with stronger CL intensities on all the array spots of C1, C2 and C3 in the presence of U1&D1, U2&D2, and U3&D3 DNA pairs (Fig. 2C and D). This was because the HCR assembly formed by H1 and H2 provided more binding sites for HRP to produce CL signal, and the double CL intensity verified the amplification ability of HCR assembly [4]. Due to the surface steric hindrance effect, here the amplification ability was lower than homogeneous HCR assembly. In addition, no bright dots were observed in sensing cells incubated with H1 or the mixture of H1 and H2 in the absence of U&D DNA pairs, indicating the good noise of CL microarray imaging assay ascribing to the low nonspecific adsorption of DNA strands as well as HRP on the microarray. Although a slight increase of the noise was observed in the assay using HCR assembly, the signal-to-noise ratio was maintained. These results indicated the HCR amplification strategy could improve the signal without the sacrifice of signal-to-noise ratio.

3.4. Assay performance

The analytical performance of CL microarray imaging assay requiring only microliter sample was investigated under the optimal conditions. In order to test the analytical performance of the proposed DNA microarray with HCR amplification, the U&D DNA pairs were firstly served as targets to be calculated by the CL microarray imaging assay. As illustrated in Fig. 3A, after the sensing cells were incubated with the mixture of U1&D1, U2&D2 and U3&D3 DNA pairs at different concentrations, the brightness of all the array spots of C1, C2 and C3 on the sensing cells increased with the increasing concentration of the three U&D DNA pairs. The CL intensities were proportional to the logarithm value of the concentrations of U1&D1, U2&D2, and U3&D3 DNA pairs, respectively, over the range of 1–500 nM.

The cross-reactivity among the array spots and nonspecific U&D DNA pairs was studied by incubating the sensing cells with U1&D1, U2&D2, and U3&D3 DNA pairs, respectively. As shown in Fig. 3B, only the array spots for the corresponding U&D DNA pair in the incubation solution showed strong brightness with high CL intensity, while other nonspecific array spots were faint with low CL intensity. Therefore, cross-reactivity at the CL microarray imaging assay was negligible, making it possible for assay of multiple targets in a single run without interfering with each other.

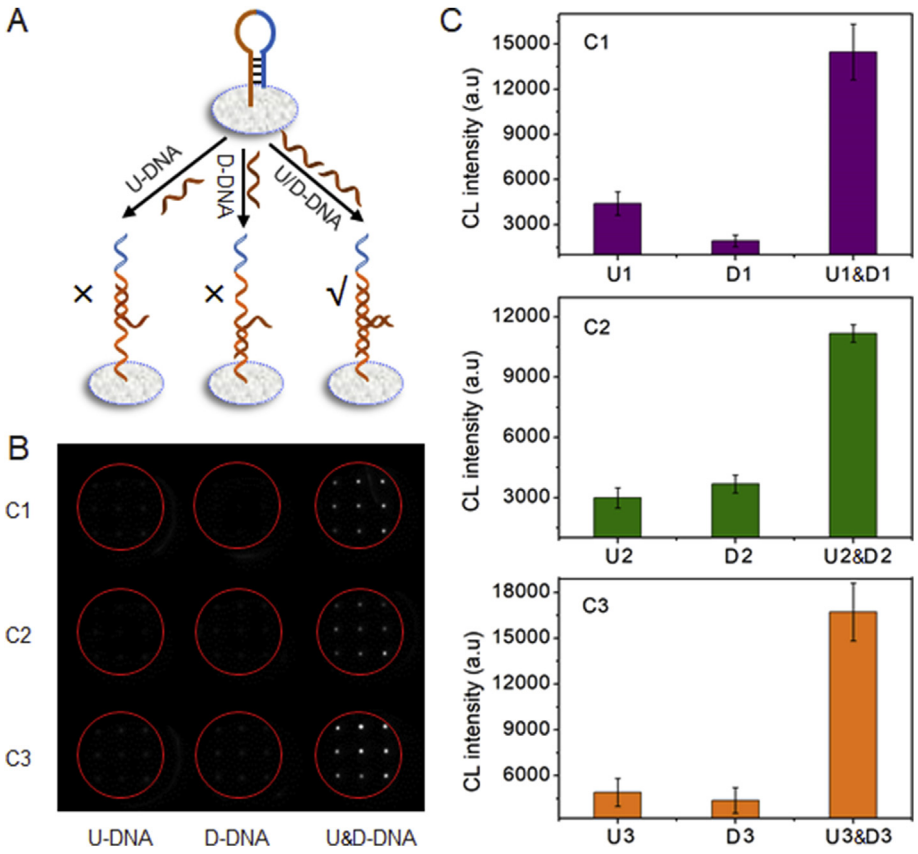


Fig. 1. (A) Illustration of the spotted hairpin DNA incubated with U-DNA, D-DNA and U&D- DNA pairs. (B) CCD image and (C) CL intensities of the array spots of C1, C2 and C3 in the presence of their corresponding U-DNA, D-DNA and U&D-DNA pairs at 500 nM, respectively.

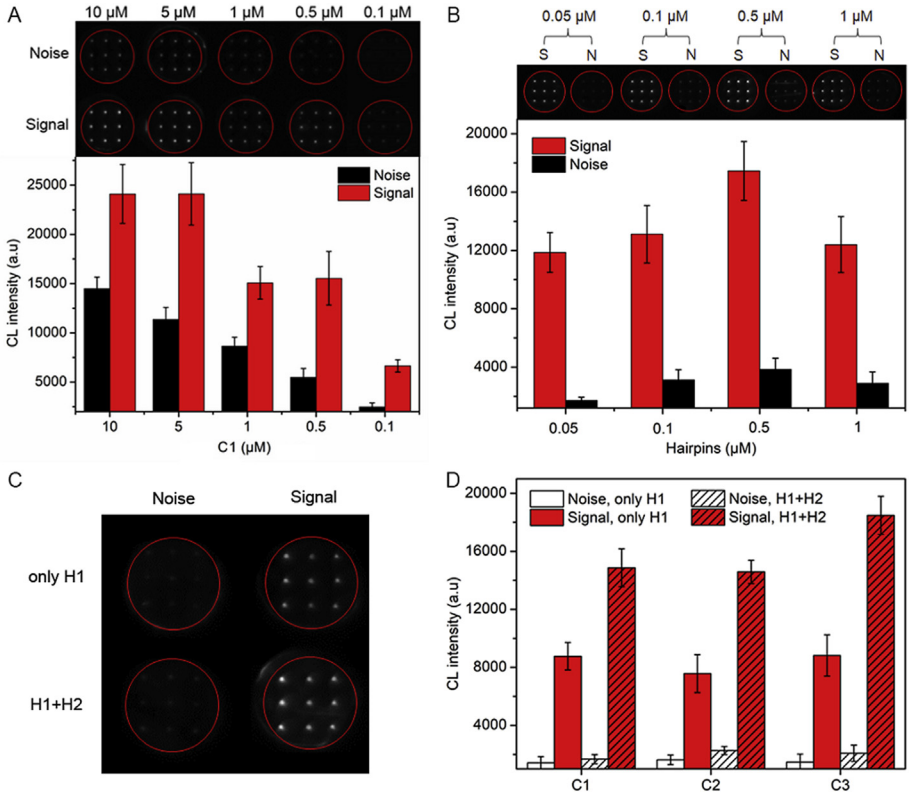


Fig. 2. Optimization of the concentrations of (A) printing hairpin DNA and (B) HCR probes on C1 spots with U1&D1 DNA pairs at 500 nM. (C) CCD image and (D) CL intensities of the array spots in sensing cells incubated with H1 or the mixture of H1 and H2 in the absence and presence of the mixture of U1&D1, U2&D2, and U3&D3 DNA pairs at 500 nM.

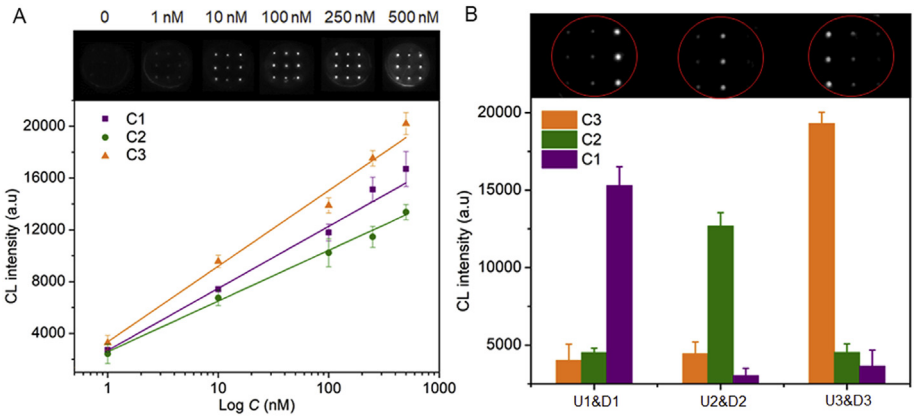


Fig. 3. (A) CCD image and the linear relationship between the CL intensities of the array spots and logarithm of concentrations of U&D DNA pairs. (B) CCD image and the CL intensities of the array spots of C1, C2 and C3 when the sensing cells incubated with 500 nM U1&D1, U2&D2, and U3&D3 DNA pairs, respectively.

3.5. Detection of proteins

The practicability of the CL microarray imaging assay was demonstrated for multiplex detection of CEA, AFP and CA 125. Here, the sensing cells with all array spots printed with C1, C2 and C3 were used to detect CEA, AFP and CA 125, respectively. These sensing cells were incubated with the mixtures containing antibody-DNA pairs and corresponding targets in different concentrations, and the CL images of array spots in all sensing cells were collected simultaneously by CCD. The brightness of the array spots on the sensing cells increased with the increasing concentrations of CEA, AFP and CA 125, respectively (Fig. 4A). The CL intensities were proportional to the logarithm value of analyte concentration over the ranges of 0.05–500 ng mL^{-1} for CEA, 0.06–600 ng mL^{-1} for AFP, and 0.02–200 U mL^{-1} for CA 125, respectively (Fig. 4B). Benefitting from DNA microarray along with the proximity binding-induced HCR amplification, the detection results of the proposed CL microarray imaging assay were superior to those of paper [38] and glass side [39]-based multiplex CL immunoassays, and comparable to those of macro array-based CL imaging assays using proximity-induced DNAzyme [40] and endonuclease-mediated cycle amplifications [41].

The specificity of the CL microarray imaging assay to target proteins was investigated. The sensing cells with all array spots printed with C1, C2 and C3 were used to study the detection specificity of CEA, AFP and CA 125, respectively (Fig. 5A). After incubating the sensing cells with different combinations of CEA, AFP and CA 125, only the array spots for the corresponding target proteins in the incubation solution showed strong brightness with high CL intensity (Fig. 5). In addition, compared with the blank (column 1), the presence of nonspecific antigens didn't produce any increase of the CL intensity (column 4). These results indicated no cross-react among the array spots and nonspecific proteins occurred, showing good specificity of the CL microarray imaging assay.

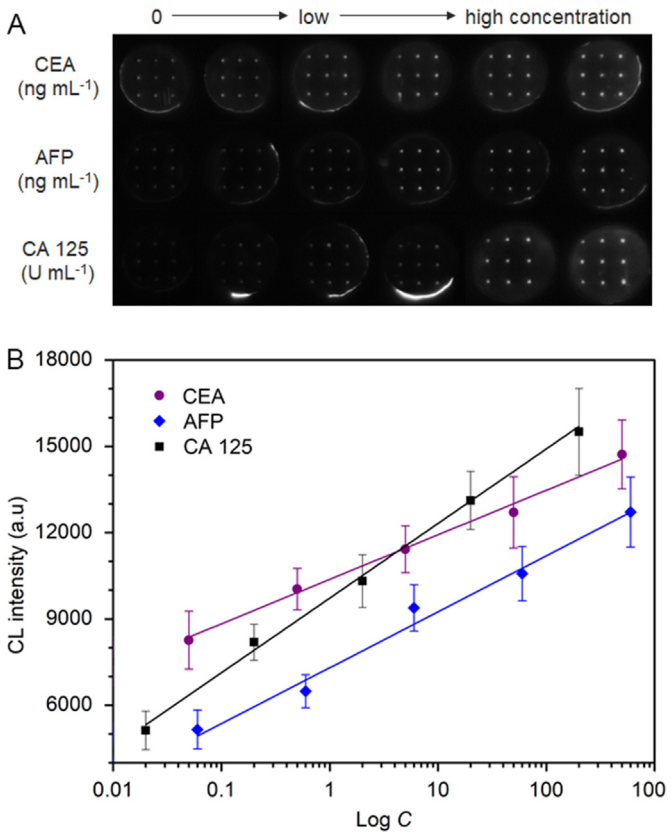


Fig. 4. (A) CCD image and (B) the linear relationship between the CL intensities of the array spots and logarithm of concentrations of CEA, AFP and CA 125.

3.6. Sample analysis

To evaluate the application potential of the proposed CL microarray imaging assay, the determination of CEA, AFP and CA 125 in clinical serum samples from cancer patients was conducted and the results were compared with commercial electrochemiluminescent assay. All the assay of three biomarkers was satisfactory with relative errors less than 9.39% (Table 1), demonstrating the CL microarray imaging assay had good reliability and was promising for clinical application. One thing to be noted, the ability for point-of-care testing (POCT) is another important index of a method for clinical application [42]. As the proposed CL microarray imaging assay included immunoreaction, HCR amplification, enzyme conjugation, and multiple washing steps, it was not suitable for POCT at the present concept. However, by combining DNA microarray, proximity-induced immunoassay with DNA walker-based surface amplification, one-step CL microarray imaging assay is under designing in our lab for the development of next generation POCT device.

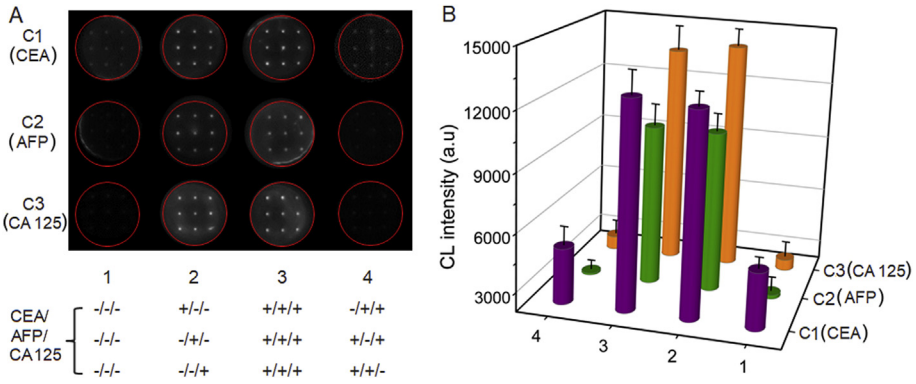


Fig. 5. (A) CCD image and (B) the CL intensities of the array spots in sensing cells with all spots printed with C1, C2 and C3 respectively. 1, 2, 3, and 4 represented the sensing cells incubated with different combinations of 50 ng mL^{-1} CEA, 60 ng mL^{-1} AFP and 20 U mL^{-1} CA 125. + and − meant the presence and absence of the corresponding protein biomarkers.

Table 1
Assay results of CEA, AFP and CA 125 in clinical serum samples using the proposed CL microarray imaging assay and reference electrochemiluminescent assay.

Biomarkers	1	2	3	4	5	
CEA (ng mL^{-1})	3.24	54.37	0.28	80.92	2.01	This work
	3.18	55.16	0.309	80.57	1.91	Reference assay
	1.89	−1.43	−9.39	0.43	5.24	Relative error (%)
AFP (ng mL^{-1})	9.62	20.06	1.53	2.62	117.24	This work
	9.87	20.41	1.44	2.59	113.7	Reference assay
	−2.53	−1.71	6.25	1.16	3.11	Relative error (%)
CA 125 (U mL^{-1})	32.36	17.72	9.65	22.61	43.52	This work
	31.85	18.16	9.82	21.85	41.63	Reference assay
	1.60	−2.42	−1.73	3.48	4.54	Relative error (%)

4. Conclusions

This work develops a CL microarray imaging assay for sensitive screen detection of multiple protein biomarkers. Different from the traditional multiplexed immunoassays based on protein macro/micro-arrays, the proposed CL imaging assay is constructed on a DNA microarray with the help of proximity binding-induced DNA assembly for signaling, offering the features of low lost, easy preparation and storage, high multiplex degree and specificity. In addition, by integrating the DNA microarray with HCR amplification, the CL microarray imaging assay shows good detection sensitivity for protein biomarkers. Moreover, the CL microarray imaging assay can be extended to screen detect a maximum of 9 protein targets in a microliter sample by designing different hairpin DNAs and antibody-DNA pairs. Overall, the CL microarray imaging assay shows advantages of good cost effectiveness, low consumption and multiple assay capability, exhibiting great promise for protein-related research and molecular disease diagnosis.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.aca.2018.05.043.

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