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Target-triggered parallel rolling circle amplification circuits for chemiluminescent imaging assay of proteins

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ABSTRACT

This work designed a strategy of target-triggered parallel rolling circle amplification circuits (PRCAC) for chemiluminescent (CL) imaging assay of proteins. The PRCAC was performed in a homogeneous system containing a pair of affinity probes, a predesigned Block/initial-Primer (Block/iPrimer) hybrid and a tailored circular-DNA/cyclic-Primer hybrid (cDNA/cPrimer). In the presence of target protein, the iPrimer was firstly released through target-initiated proximity binding-induced strand displacement to recognize cDNA/cPrimer, which led to an iPrimer-activated rolling circle amplification (iRCA) and the release of cPrimer to recognize cDNA/cPrimer for triggering self-feeding multiple cPrimer-activated RCAs. The PRCAC could generate numerous G-quadruplex units for forming DNAzyme, which catalyzed the oxidation of luminol by H_2O_2 to produce strong CL signal, and thus led to an amplified CL protein imaging method. This method could detect both antigen and antibody through changing the specific affinity probes. Using NT-proBNP and anti-PCSK9 antibody as analytes, this method exhibited the linear ranges of 0.01–10 pg mL^{-1} and 0.1–1000 pg mL^{-1}, respectively. The low limits of detection, excellent specificity, simple operation, relatively short analytical time and acceptable reliability demonstrated the applicability of the PRCAC in biomarker analysis.

1. Introduction

Rapid and sensitive detection of protein biomarkers is very important in clinic diagnostics of acute diseases, such as acute myocardial infarction and acute heart failure, for performing effective treatment within 2 h [1,2]. The general enzyme-linked immunosorbent assay (ELISA) is unsuitable for this purpose due to multiple time-consuming washing and incubation steps [3]. The popular engineered solutions include lateral flow immunoassay (LFIA) [4–6] and proximity binding-based immunoassay (PBIA) [7,8]. However, the lacks of quantification ability and sensitivity limit LFIA application in rapid analysis of protein biomarkers [4]. Different from LFIA, PBIA is a homogeneous assay technology and can combine with polymerase chain reaction [9,10], rolling circle amplification (RCA) [11,12], exonuclease/endonuclease-mediated cycle amplification [13], hybridization chain reaction (HCR) [14], and catalytic hairpin assembly (CHA) [15] to achieve highly sensitive fluorescent [11,12,15], electrochemical [16], and chemiluminescent (CL) [13,14] detections of protein biomarkers.

CL is an ideal clinic measurement technology due to its advantages of low background, no interference of autofluorescence, high sensitivity, easy operation, cheap and simple instruments (no need of external light sources) [17–19]. The CL signal can be generated through horseradish peroxidase-mimicking DNAzyme catalyzed oxidation of luminol by H_2O_2 [19,20], which provides an opportunity to greatly improve the sensitivity of CL detection by combining with RCA, HCR or CHA to in-site form numerous G-quadruplex/hemin (G4/hemin) units [19,21–23]. RCA is a typical isothermal enzymatic DNA amplification technique. It can amplify a short primer oligonucleotide to a long DNA chain containing tens of hundreds of repetitive sequences that are complementary to the template. Due to its high amplification efficiency, RCA is often used for signal amplification in DNAzyme based CL assays [24–28]. In order to achieve the rapid and sensitive detection of protein biomarkers, this work designed a strategy of target-triggered parallel rolling circle amplification circuits (PRCAC) to amplify DNAzyme formation. The PRCAC consisted of an initial-Primer-activated RCA (iRCA) and multiple cyclic-Primer-activated RCAs (cRCAs), and was achieved in a homogeneous system containing a pair of affinity probes, a predesigned Block/initial-Primer (Block/iPrimer) hybrid and a tailored circular-DNA/cyclic Primer hybrid (cDNA/cPrimer). Upon the

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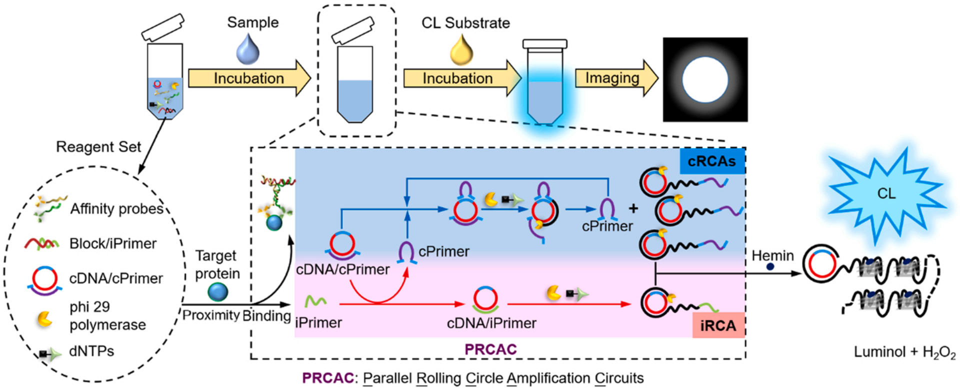

Scheme 1. Schematic illustration of CL lmaging assay of protein via target-triggered PRCAC.

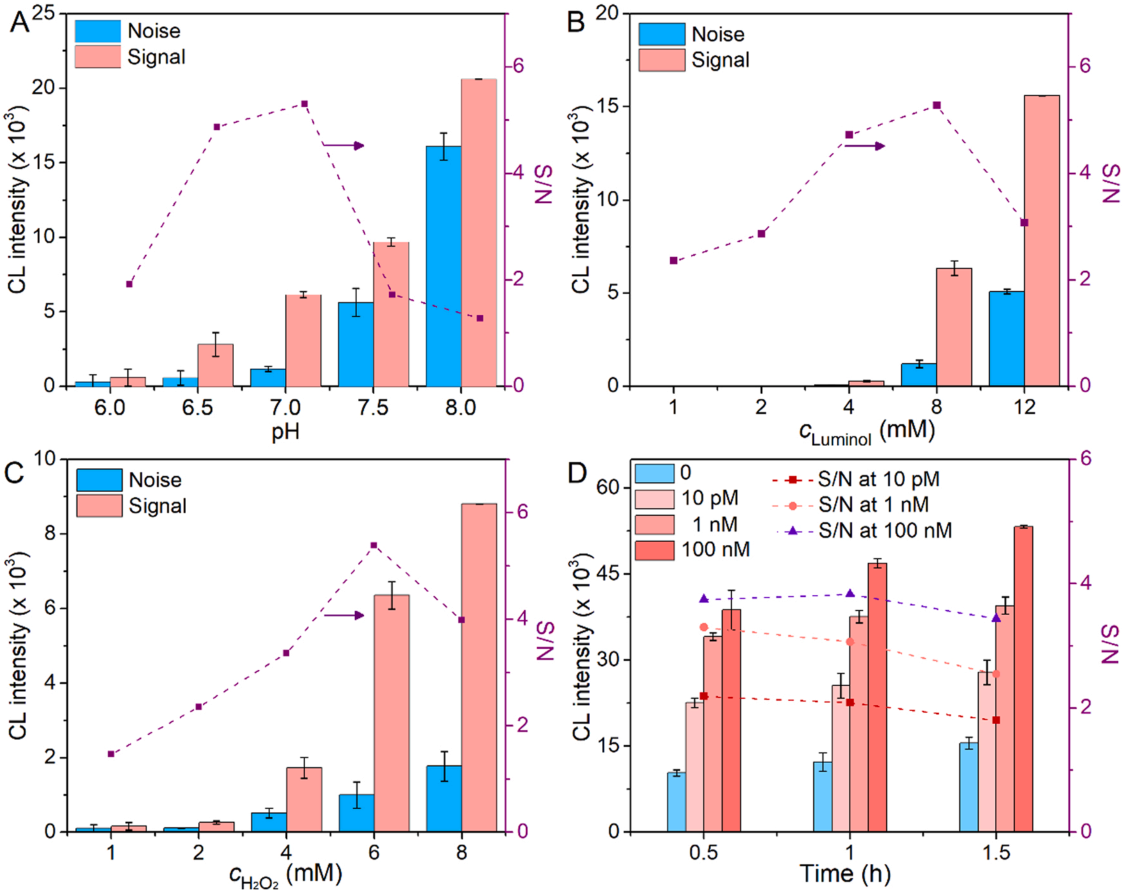

Fig. 1. Effects of (A) pH, (B) luminol concentration and (C) H_2O_2 concentration on CL intensity of 10 mM PBS containing luminol, H_2O_2 and 20 nM hemin in absence (noise) or presence (signal) of 40 nM G4 DNA. When one condition was optimized, other conditions were at pH 7.0, 8 mM luminol or 6 mM H_2O_2. (D) CL intensity of 10 mM pH 7.0 PBS containing 8 mM luminol, 6 mM H_2O_2, 20 nM hemin, and PRCAC product obtained after incubating the mixture of 5 nM cDNA/cPrimer, 0.05 U μL^{-1} phi 29, 0.2 mM dNTPs, 0.1 mM BSA and iPrimer at 0 (noise), 0.01, 1 and 100 nM (signal) for 0.5-h, 1-h and 1.5-h incubation ($n = 3$).

recognition of the target protein to the affinity probes, a proximity binding-induced strand displacement was initiated to release iPrimer from Block/iPrimer, which led to an iPrimer-activated RCA (iRCA) and the release of cPrimer to further recognize the cDNA/cPrimer for triggering the self-feeding cRCAs (Scheme 1). The designed PRCAC could produce numerous G-quadruplex units in a relatively short time (30 min), thus after the addition of hemin, abundant horseradish peroxidase-mimicking DNAzyme could be formed for highly sensitive CL imaging assay of protein biomarkers.

To demonstrate the feasibility of the designed PRCAC and proposed CL imaging assay method, this work used aminoterminal pro-brain natriuretic peptides (NT-proBNP), an antigen biomarker of heart failure [2], and anti-proprotein convertase subtilisin/kexin type 9 antibody (anti-PCSK9 antibody), an antibody drug for cardiovascular disease, as analyte models to design two pairs of affinity probes. The proposed CL imaging method achieved the detection limit of fg mL^{-1} level and wide concentration ranges. The high sensitivity, short analytical time, simple operation, and good versatility showed the applicability of the PRCAC in sensitive, rapid and high-throughput assay of proteins biomarkers, and POCT diagnosis of acute diseases.

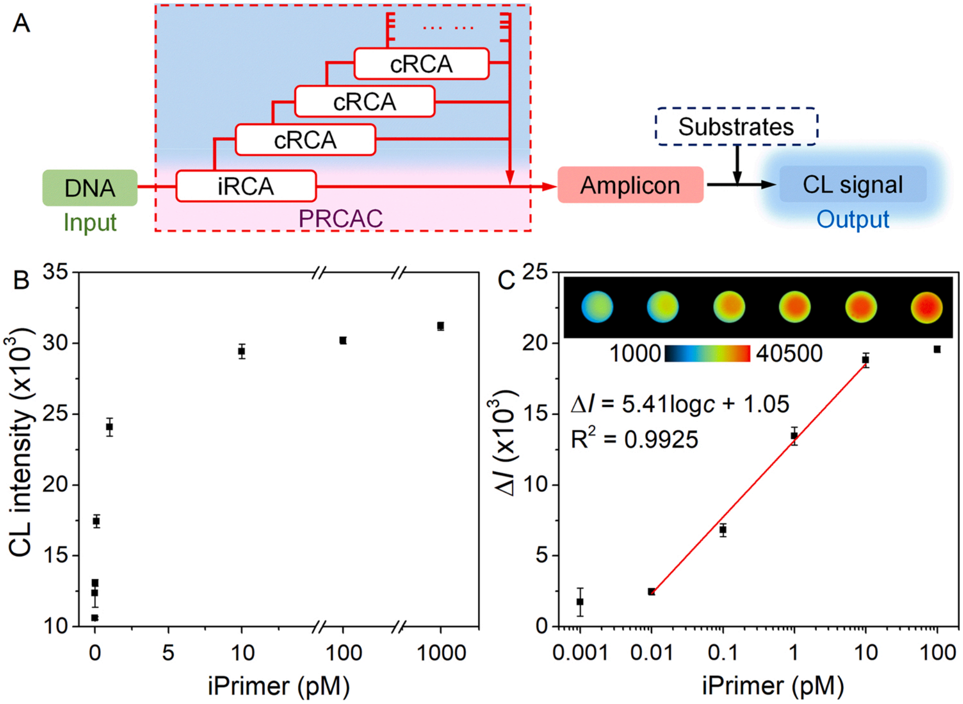

Fig. 2. (A) Structural diagram of DNA-initiated PRCAC for CL imaging assay. (B) CL intensity and (C) relative CL signal of 10 mM pH 7.0 PBS containing 8 mM luminol, 6 mM H_2O_2, 20 nM hemin and PRCAC product as logarithm function of iPrimer concentration after incubating 5 nM cDNA/cPrimer, 0.05 U μL^{-1} phi 29, 0.2 mM dNTPs, 0.1 mM BSA and iPrimer for 30 min at 37 °C (n = 3).

2. Experimental

2.1. General experiments

Materials, reagents, apparatus, preparation of cDNA/cPrimer hybrid, synthesis of affinity probes, electrophoresis analysis, CL response to iPrimer, and FL response PRCAC to iPrimer are presented in the Supplementary material.

2.2. CL imaging assay of protein

0.5 μL sample or target protein in PBS (10 mM, pH 7.0) or complex medium (10% human serum or DMEM) was mixed with 4.5 μL reagent set to incubate for 30 min at 37 °C. Here, the reagent set was freshly prepared, which included affinity probe pairs (10 ng mL^{-1} respectively), Block/iPrimer (50 nM), cDNA/cPrimer (50 nM), phi 29 polymerase (0.5 U μL^{-1}), dNTPs (2 mM), BSA (1 mM) and 1 ×phi 29 reaction buffer. Then, 10 μL hemin (0.1 μM in 5 ×PBS-3) was added to react 10 min, and the CL image was collected immediately after the addition of 35 μL freshly prepared CL substrate (15 μL of 20 mM H_2O_2 and 20 μL of 20 mM luminol).

3. Results and discussion

3.1. CL imaging assay via target-triggered PRCAC

The target-triggered PRCAC was performed with a reagent set (Scheme 1). The iPrimer contained 28 bases complementary to cDNA, while the cDNA contained an extra fragment complementary to two terminal regions of cPrimer (Table S1, blue regions), each with 5 bases pairs. The cDNA/cPrimer was prepared via the ligation of a circular padlock with cPrimer (Table S1, purple regions). The presence of endmost protruding sites between cDNA and cPrimer prevented the RCA itself [29]. After cDNA/cPrimer was recognized by iPrimer to form cDNA/iPrimer, the iRCA process was activated, and the released cPrimer hybridized with its complementary sequence in another cDNA/cPrimer to induce a self-feeding cRCA (Fig. S1). The PRCAC produced a large amount of G4 duplicate sequences in a relatively short time.

The CL imaging assay was simply conducted by adding a sample to the reagent set, which led to target-initiated proximity binding through the recognition of target protein to a pair of affinity probes to form a proximity-ligated complex. The complex contained a sequence complementary to Block (red regions of HS-DNA1 and HS-DNA2), and could induce the strand displacement to release iPrimer from Block/iPrimer hybrid (Fig. S2). Thus the proximity binding-induced strand displacement could be considered as a DNA input for triggering the PRCAC, which produced abundant G4/hemin DNAzyme units in the presence of hemin for sensitive CL imaging assay of target protein.

3.2. Design of cDNA/cPrimer for PRCAC

The cPrimer was used to prepare cDNA via its hybridization with padlock and following ligation reaction, which generated cDNA/cPrimer (Fig. S3A). To prevent the RCA of cDNA/cPrimer itself, two terminal overhang regions were designed in cPrimer. The padlock contained a sequence complementary to the terminal overhang regions. Thus the cPrimer released from cDNA/cPrimer could hybridize with cDNA/cPrimer to release more cPrimer, leading to a self-feeding cRCAs (Fig. S1). By using FAM-cPrimer-6-BHQ as the cPrimer, the hybridization reactions were verified by a fluorescence resonance energy transfer (FRET) experiment (Fig. S3B). The hybridization complex of cPrimer with padlock showed the same fluorescence intensity, while the complex of cPrimer' with the complementary sequence designed in padlock for cRCAs showed decreased fluorescence intensity due to the approach of FAM to BHQ. These results also indicated that cPrimer preferred to hybridize with padlock through the intermediate ligation region due to the stable hybridization product of the consecutive complementary sequence of cPrimer with padlock. However, when this hybridization was saturated, the excess cPrimer would hybridize with padlock through its terminal overhang regions.

The sequence of cPrimer was optimized by electrophoretic analysis

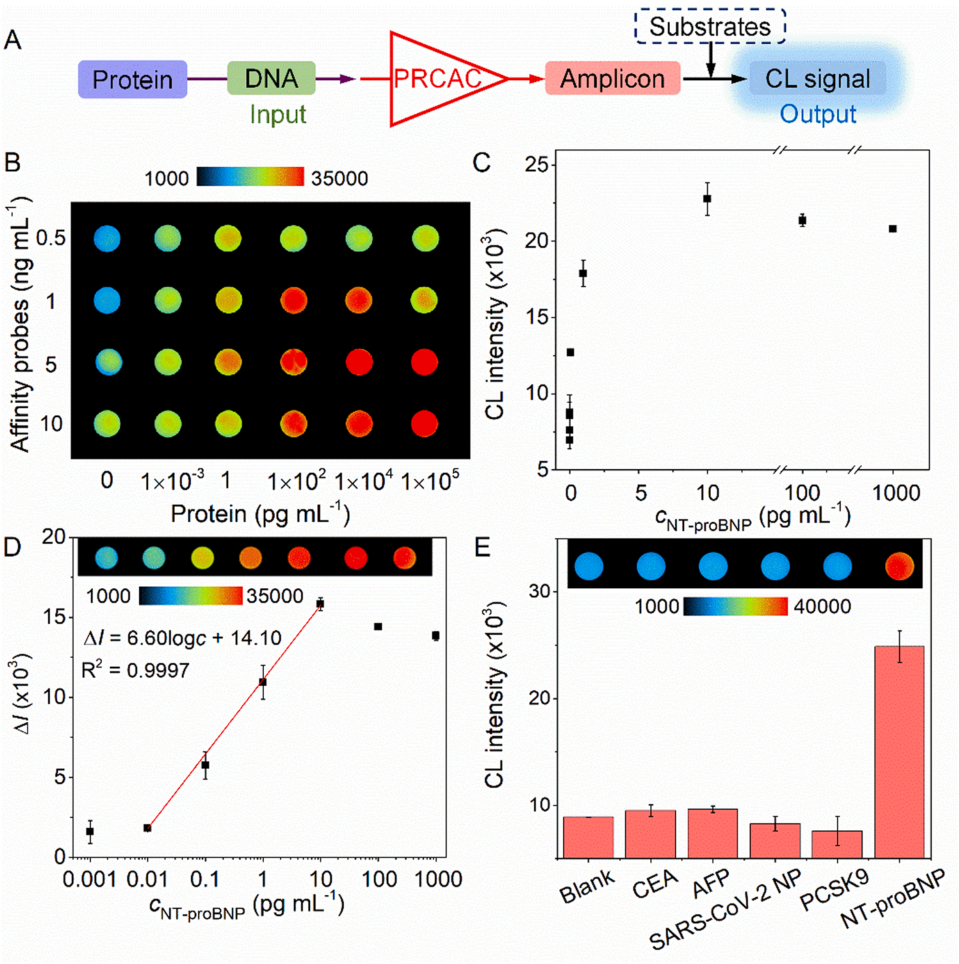

Fig. 3. (A) Structural diagram of protein-initiated PRCAC for CL imaging assay. (B) CL image responding to NT-proBNP at different concentrations with marked concentrations of DNA1-NT-proBNP antibody from T6 and DNA2-NT-proBNP antibody from T9. (C) CL intensity and (D) relative CL signal as logarithm function of NT-proBNP concentration. (E) CL intensity of 10 pg mL^{-1} CEA, AFP, SARS-CoV-2 NP, PCSK9, and NT-proBNP proteins (n = 3). The CL image and CL signals were recorded in 10 mM pH 7.0 PBS containing 8 mM luminol, 6 mM H_2O_2, 20 nM hemin, and PRCAC product obtained by incubating 1 ng mL^{-1} DNA1-NT-proBNP antibody from T6, 1 ng mL^{-1} DNA2-NT-proBNP antibody from T9, 5 nM cDNA/cPrimer, 5 nM Block/iPrimer, 0.05 U μL^{-1} phi 29, 0.2 mM dNTPs, 0.1 mM BSA and NT-proBNP at different concentrations for 30 min at 37 °C (n = 3).

of the hybridization products of padlock with cPrimer-1 to cPrimer-9 (Table S1). After incubation and ligation, only the mixture of cPrimer-6 and padlock showed a single band (Fig. S4), indicating the successful synthesis of cDNA/cPrimer hybrid with high purity and good stability. Thus cPrimer-6 with 16 bases complementary to padlock for cDNA synthesis and 5 bases at each terminal region complementary to padlock for PRCAC was selected to prepare cDNA/cPrimer hybrid. The fluorescence assay further verified that the PRCAC was only initiated by iPrimer (Fig. S5).

3.3. Optimization of detection conditions

The detection conditions such as pH, luminol concentration and H_2O_2 concentration were optimized by replacing PRCAC product with G4 DNA to obtain the CL signal. The maximum signal-to-noise ratio occurred at pH 7.0 (Fig. 1A). At 8 mM luminol and 6 mM H_2O_2, the signal-to-noise ratios showed the maximum values, respectively (Fig. 1B and C), which were thus used in CL imaging. In addition, the reaction time of PRCAC was a vital parameter since it determined both the length and the number of the produced chain with repetitive G4 units. The longer reaction time could produce more G4 units for G4/hemin DNAzyme formation, which led to stronger CL intensity. However, the increasing incubation from 30 to 90 min did not obviously improve the signal-to-noise ratio (Fig. 1D). This could be attributed to the presence of few cPrimer in the obtained cDNA/cPrimer due to the uncomplete hybridization of cPrimer with padlock from the point of view of thermodynamic equilibrium, which would lead to the increase of background. Fortunately, the increasing CL intensity in the absence of iPrimer (noise) did not affect the CL detection of protein when 30 min was selected as the optimal PRCAC time, because the CL intensity or signal-to-noise ratio showed a positive relativity with iPrimer concentration (Fig. 1D), which was stoichiometric to the protein analyte.

3.4. CL performance of PRCAC

In PRCAC, iPrimer could simultaneously initiate iRCA and cRCAs to produce multiple DNA chains consisting of numerous G4 fragments (Fig. S1). Therefore, PRCAC possessed stronger amplification ability than common RCA, in which one iPrimer produces one long DNA chain [28]. The superior amplification ability of PRCAC was verified by electrophoretic analysis (Fig. S6A) and higher CL response (Fig. S6B).

The analytical performance of PRCAC was firstly evaluated by using iPrimer as the DNA input to trigger the PRCAC for amplicon production (Fig. 2A). As expected, the CL intensity increased with the increasing concentration of iPrimer (Fig. 2B). The plot of the relative CL response versus the logarithm of iPrimer concentration ranging from 0.01 to 10 pM showed good linearity (Fig. 2C). This result guaranteed the feasibility of quantitative analysis for protein detection through PRCAC combined with proximity binding-induced strand displacement.

3.5. CL imaging assay of NT-proBNP

To verify the application of PRCAC in CL imaging assay of protein target, a pair of affinity probes specific to NT-proBNP were used to form a proximity-ligated complex. The released iPrimer as a DNA input then initiated the PRCAC to produce G4 amplicon for CL output (Fig. 3A). Here, the affinity probes were prepared with a pair of anti-NT-proBNP antibodies (Fig. S7).

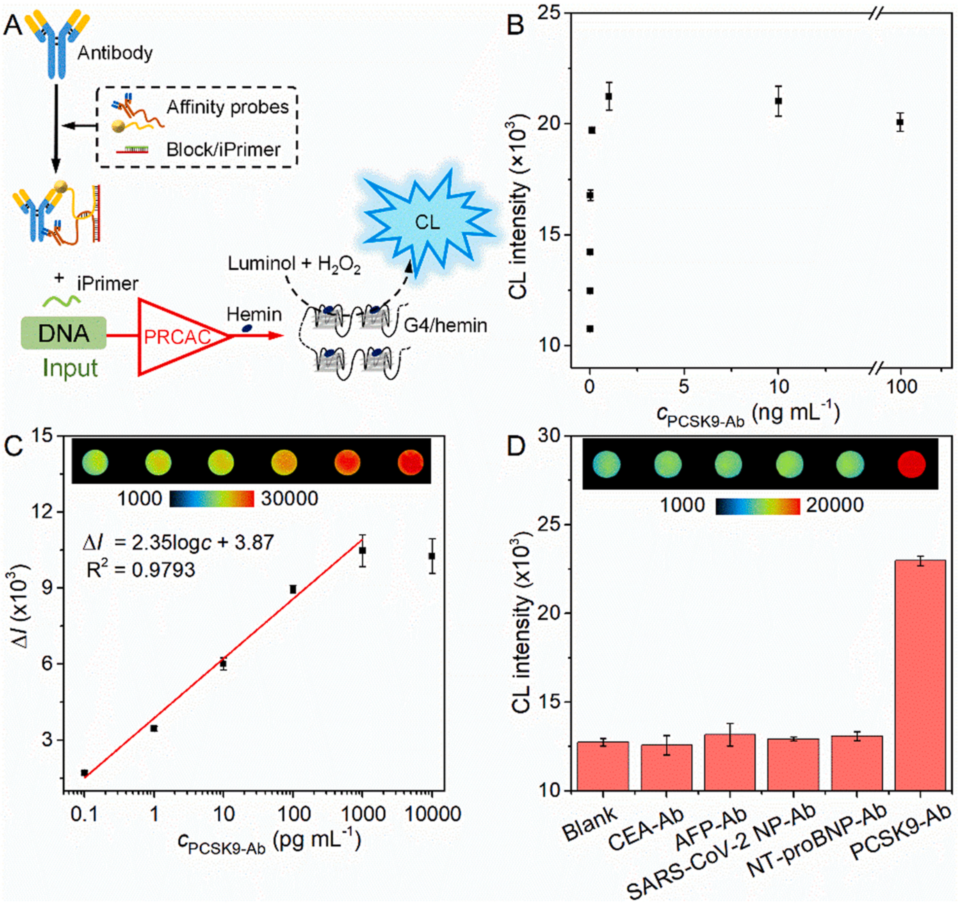

Fig. 4. (A) Schematic diagram of CL imaging assay of antibody. (B) CL intensity and (C) relative CL signal as logarithm function of anti-PCSK9 antibody concentration. The CL image and CL signals were recorded in 10 mM pH 7.0 PBS containing 8 mM luminol, 6 mM H_2O_2, 20 nM hemin, and PRCAC product obtained by incubating 1 ng mL^{-1} DNA1-PCSK9, 1 ng mL^{-1} DNA2-Ab2, 5 nM cDNA/cPrimer, 5 nM Block/iPrimer, 0.05 U μL^{-1} phi 29, 0.2 mM dNTPs, 0.1 mM BSA and anti-PCSK9 antibody at different concentrations for 30 min at 37 °C ($n = 3$). (D) Specificity of CL imaging assay at 1 ng mL^{-1} anti-CEA, anti-AFP, anti-SARS-CoV-2 NP, anti-NT-proBNP and anti-PCSK9 antibody ($n = 3$).

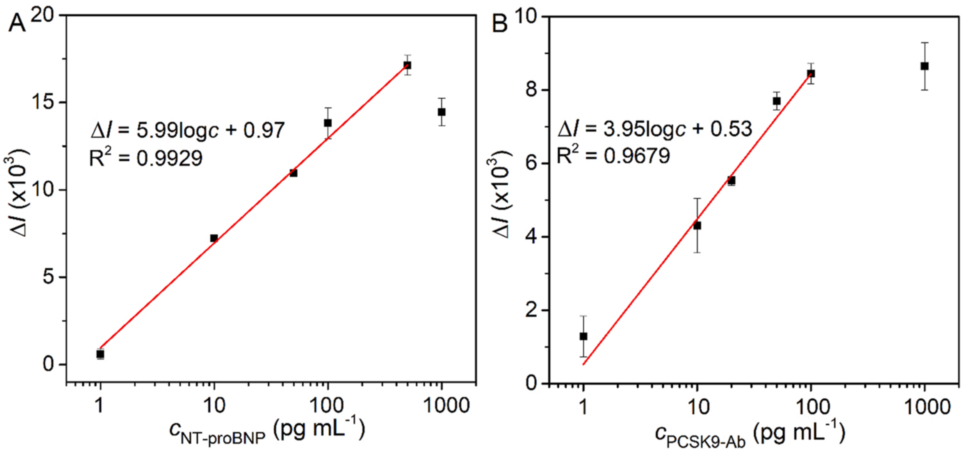

Fig. 5. Calibration curves of (A) NT-proBNP in human serum and (B) anti-PCSK9 antibody in DMEM culture medium.

The concentrations of affinity probes were optimized by detecting NT-proBNP at the concentration ranging from 0 to 100 ng mL^{-1} (Fig. 3B). When the concentrations of probes were lower than 1 ng mL^{-1}, the detectable concentration range was narrow, while high probe concentration led to greater background. Thus 1 ng mL^{-1} was selected as the optimal probe concentration, at which lower detection limit with the same upper limit of the linear calibration range could be obtained.

Under the optimal conditions, the CL intensity increased and trended to the maximum value with the increasing NT-proBNP concentration (Fig. 3C). When NT-proBNP concentration was higher than 10 pg mL^{-1}, the CL signal decreased due to the Hook effect [30]. The plot of relative CL response versus the logarithm of NT-proBNP concentration showed good linearity in the range of 0.01–10 pg mL^{-1} (Fig. 3D). The limit of detection was calculated to be 3.59 fg mL^{-1} (at 3σ), which was much lower than previous assays with other amplification strategies (Table S2). In addition, the specificity of the proposed CL imaging assay for NT-proBNP was also verified by comparing the CL signals responding to different proteins, such as CEA, AFP, SARS-CoV-2 NP, and PCSK9. As expected, only the sample containing NT-proBNP showed a distinct signal (Fig. 3E), indicating the good selectivity of this PRCAC based CL imaging assay.

3.6. CL imaging assay of PCSK9 antibody

The proposed CL imaging assay could be extended to detect other protein targets, such as antibody, by changing the affinity probes. Here, anti-PCSK9 antibody was used to verify its extendibility. A pair of affinity probes, DNA1-PCSK9 and DNA2-Ab2, were used to form a sequence for proximity binding-induced strand displacement to perform the DNA input of PRCAC (Fig. 4A). Similarly, the CL intensity increased with the increasing anti-PCSK9 antibody concentration and then decreased after 1 ng mL^{-1} due to the Hook effect (Fig. 4B). The linear calibration range was from 0.1 pg mL^{-1} to 1 ng mL^{-1} (Fig. 4C). Anti-CEA, anti-AFP, anti-SARS-CoV-2 NP and anti-NT-proBNP antibody did not show significant CL response (Fig. 4D), indicating good detection specificity of this CL imaging assay.

3.7. Sample analysis

To evaluate the clinical application of the proposed CL imaging assay method, NT-proBNP spiked in human serum samples and anti-PCSK9 antibody spiked in cell culture medium DMEM were detected with the corresponding calibration curves (Fig. 5). Obviously, the calibration curves of both NT-proBNP in serum (Fig. 5A) and PCSK9-Ab in DMEM (Fig. 5B) were different from these of proteins in PBS (Fig. 3D and Fig. 4C), indicating that the detection of target protein in different solutions required the corresponding calibration curve due to the influence of complex matrix. From the calibration curves shown in Fig. 5, NT-proBNP in serum and PCSK9-Ab in culture medium were detected, respectively. The recovery ranged from 87.1 % to 107.6 % (Table S3), which indicated the acceptable reliability of the proposed CL imaging assay. In order to further improve the detection accuracy, more samples and condition optimization should be performed, and the repeatability of operation also should be improved.

4. Conclusion

A sensitive CL imaging assay method for protein targets has been proposed with a newly designed target-triggered parallel rolling circle amplification circuits (PRCAC). The PRCAC is triggered via the recognition of cDNA/cPrimer by iPrimer, which is released from Block/iPrimer through target-induced proximity binding and following strand displacement. It uses a reagent set to perform parallel iRCA and multiple cRCAs for rapidly producing numerous G4/hemin DNAzyme and greatly amplifying the CL signal of luminol-H_2O_2 system. At a relatively short PRCAC time (30 min), the proposed method achieves the detection limits of fg mL^{-1} to tens fg mL^{-1} level and acceptable concentration range along with good specificity. By changing the affinity probes for target-induced proximity binding, the designed PRCAC can be extended for different protein targets, which has been verified by using NT-proBNP and anti-PCSK9 antibody as the targets. However, this method requires two-step incubations due to the instability of hemin in aqueous solution, and the intrinsic catalytic property of hemin and RCA amplification also lead to relatively high background. Future work needs to develop stable and sensitive DNAzyme switch. To sum up, both the PRCAC and the proposed CL imaging assay method possess good prospect in sensitive, rapid and high-throughput assay of proteins biomarkers.

CRediT authorship contribution statement

Hang Ao: Conceptualization, Methodology, Data curation, Writing-original draft. **Wencheng Xiao** and **Yuhui Chen:** Formal analysis. **Jie Wu:** Conceptualization, Methodology, Writing. **Huangxian Ju:** Conceptualization, Funding acquisition, 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.

Data availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2023.133579.

References

[1] B.R. Brodie, C. Hansen, T.D. Stuckey, S. Richter, D.S. VerSteeg, N. Gupta, W. E. Downey, M. Pulsipher, Door-to-balloon time with primary percutaneous coronary intervention for acute myocardial infarction impacts late cardiac mortality in high-risk patients and patients presenting early after the onset of symptoms, J. Am. Coll. Cardiol. 47 (2006) 289–295.

[2] T.A. McDonagh, M. Metra, M. Adamo, R.S. Gardner, A. Baumbach, M. Böhm, H. Burri, J. Butler, J. Celutkien, O. Chioncel, J.G.F. Cleland, A.J.S. Coats, M. G. Crespo-Leiro, D. Farmakis, M. Gilard, S. Heymans, ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: developed by the task force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC) with the special contribution of the Heart Failure Association (HFA) of the ESC, Eur. Heart J. 42 (2021) (2021) 3599–3726.

[3] S.S. Pierangeli, E.N. Harris, A protocol for determination of anticardiolipin antibodies by ELISA, Nat. Protoc. 3 (2008) 840–848.

[4] D. Wang, S.G. He, X.H. Wang, Y.Q. Yan, J.Z. Liu, S.M. Wu, S.G. Liu, Y. Lei, M. Chen, L. Li, J.L. Zhang, L.W. Zhang, X. Hu, X.H. Zheng, J.W. Bai, Y.L. Zhang, Y.T. Zhang, M.X. Song, Y.G. Tang, Rapid lateral flow immunoassay for the fluorescence detection of SARS-CoV-2 RNA, Nat. Biomed. Eng. 4 (2020) 1150–1158.

[5] R. Chen, C.P. Ren, M. Liu, X.P. Ge, M.S. Qu, X.B. Zhou, M.F. Liang, Y. Liu, F.Y. Li, Early detection of SARS-CoV-2 seroconversion in humans with aggregation-induced nearinfrared emission nanoparticle-labeled lateral flow immunoassay, ACS Nano 15 (2021) 8996–9004.

[6] J. Wang, C.X. Jiang, J.N. Jin, L. Huang, W.B. Yu, B. Su, J. Hu, Ratiometric fluorescent lateral flow immunoassay for point-of-care testing of acute myocardial infarction, Angew. Chem. Int. Ed. 60 (2021) 13042–13049.

[7] H.Q. Zhang, F. Li, B. Dever, X.F. Li, X.C. Le, DNA-mediated homogeneous binding assays for nucleic acids and proteins, Chem. Rev. 113 (2013) 2812–2841.

[8] H.Q. Zhang, F. Li, B. Dever, C. Wang, X.F. Li, X.C. Le, Assembling DNA through affinity binding to achieve ultrasensitive protein detection, Angew. Chem. Int. Ed. 52 (2013) 10698–10705.

[9] M. Gullberg, S.M. Gustafsdottir, E. Schallmeiner, J. Jarvius, M. Bjarnegard, C. Betsholtz, U. Landegren, S. Fredriksson, Cytokine detection by antibody-based proximity ligation, Proc. Natl. Acad. Sci. USA 101 (2004) 8420–8424.

[10] L.M. McGregor, D.J. Gorin, C.E. Dumelin, D.R. Liu, Interaction-dependent PCR: identification of ligand-target pairs from libraries of ligands and libraries of targets in a single solution-phase experiment, J. Am. Chem. Soc. 132 (2010) 15522–15524.

[11] O. Söderberg, M. Gullberg, M. Jarvius, K. Ridderstråle, K. Leuchowius, J. Jarvius, K. Wester, P. Hydbring, F. Bahram, L. Larsson, U. Landegren, Direct observation of individual endogenous protein complexes in situ by proximity ligation, Nat. Methods 3 (2006) 995–1000.

[12] I. Weibrecht, E. Lundin, S. Kiflemariam, M. Mignardi, I. Grundberg, C. Larsson, B. Koos, M. Nilsson, O. Söderberg, In situ detection of individual mRNA molecules and protein complexes or post-translational modifications using padlock probes combined with the in situ proximity ligation assay, Nat. Protoc. 8 (2013) 355–372.

[13] C. Zong, J. Wu, M.M. Liu, L.L. Yang, L. Liu, F. Yan, H.X. Ju, Proximity hybridization-triggered signal switch for homogeneous chemiluminescent bioanalysis, Anal. Chem. 86 (2014) 5573–5578.

[14] Q. Xiao, J. Wu, P.Y. Dang, H.X. Ju, Multiplexed chemiluminescence imaging assay of protein biomarkers using DNA microarray with proximity binding-induced hybridization chain reaction amplification, Anal. Chim. Acta 1032 (2018) 130–137.

[15] F. Li, H.Q. Zhang, Z.X. Wang, X.K. Li, X.F. Li, X.C. Le, Dynamic DNA assemblies mediated by binding-induced DNA strand displacement, J. Am. Chem. Soc. 135 (2013) 2443–2446.

[16] M. Rossetti, S. Brannetti, M. Mocenigo, B. Marini, R. Ippodrino, A. Porchetta, Harnessing effective molarity to design an electrochemical DNA-based platform for clinically relevant antibody detection, Angew. Chem. Int. Ed. 59 (2020) 14973–14978.

[17] L. Fang, L. Guo, Z.M. Li, J.B. He, H. Cui, Temporal-spatial-color multiresolved chemiluminescence imaging for multiplex immunoassays using a smartphone coupled with microfluidic chip, Anal. Chem. 92 (2020) 6827–6831.

[18] J. Zhang, J.L. Wai, R.J. Lake, S.Y. New, Z.K. He, Y. Lu, DNAzyme sensor uses chemiluminescence resonance energy transfer for rapid, portable, and ratiometric detection of metal ions, Anal. Chem. 93 (2021) 10834–10840.

[19] Y.D. Sun, L. Shi, Q.W. Wang, L. Mi, T. Li, Spherical nucleic acid enzyme (SNAzyme) boosted chemiluminescence miRNA imaging using a smartphone, Anal. Chem. 91 (2019) 3652–3658.

[20] C. Zong, J. Wu, M.M. Liu, L.L. Yang, F. Yan, H.X. Ju, Chemiluminescence imaging for a protein assay via proximity-dependent DNAzyme formation, Anal. Chem. 86 (2014) 9939–9944.

[21] H. Fujita, Y. Kataoka, S. Tobita, M. Kuwahara, N. Sugimoto, Novel one-tube-one-step real-time methodology for rapid transcriptomic biomarker detection: signal amplification by ternary initiation complexes, Anal. Chem. 88 (2016) 7137–7144.

[22] K.L. Yang, M. Huo, Y.H. Guo, Y.Z. Yang, J. Wu, L. Ding, H.X. Ju, Target-induced cyclic DNAzyme formation for colorimetric and chemiluminescence imaging assay of protein biomarkers, Analyst 142 (2017) 3740–3746.

[23] M.M. Ali, F. Li, Z.Q. Zhang, K.X. Zhang, D.K. Kang, J.A. Ankrum, X.C. Le, W. A. Zhao, Rolling circle amplification: a versatile tool for chemical biology, materials science and medicine, Chem. Soc. Rev. 43 (2014) 3324–3341.

[24] K.E. Shopsowitz, Y.H. Roh, Z.J. Deng, S.W. Morton, P.T. Hammond, RNAi-microsponges form through self-assembly of the organic and inorganic products of transcription, Small 10 (2014) 1623–1633.

[25] F. Wang, C.H. Lu, X.Q. Liu, L. Freage, I. Willner, Amplified and multiplexed detection of dna using the dendritic rolling circle amplified synthesis of dnazyme reporter units, Anal. Chem. 86 (2014) 1614–1621.

[26] L.L. Yao, X.Q. Yu, R.J. Yu, Y.J. Zhao, A.P. Fan, Label-free chemiluminescent strategy for highly selective and sensitive detection of adenosine triphosphate by cofactor-dependent enzymatic ligation-triggered polymerase chain reaction, Sens. Actuator B-Chem. 238 (2017) 175–181.

[27] J.M. Wang, S.F. Mao, H.F. Li, J.M. Lin, Multi-DNAzymes-functionalized gold nanoparticles for ultrasensitive chemiluminescence detection of thrombin on microchip, Anal. Chim. Acta 1027 (2018) 76–82.

[28] R. Zhang, J. Wu, H. Ao, J.L. Fu, B. Qiao, Q. Wu, H.X. Ju, A rolling circle-amplified G-quadruplex/hemin DNAzyme for chemiluminescence immunoassay of the SARS-CoV-2 protein, Anal. Chem. 93 (2021) 9933–9938.

[29] C. Larsson, J. Koch, A. Nygren, G. Janssen, A.K. Rapp, U. Landegren, M. Nilsson, In situ genotyping individual DNA molecules by target-primed rolling-circle amplification of padlock probes, Nat. Methods 1 (2004) 227–232.

[30] W. Chen, S. Shan, J. Peng, D. Liu, J. Xia, B. Shao, W. Lai, Sensitive and hook effect–free lateral flow assay integrated with cascade signal transduction system, Sens. Actuator B-Chem. 321 (2020), 128465.

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