analytical chemistry

Article

Cite This: *Anal. Chem.* 2020, 92, 884−891

pubs.acs.org/ac

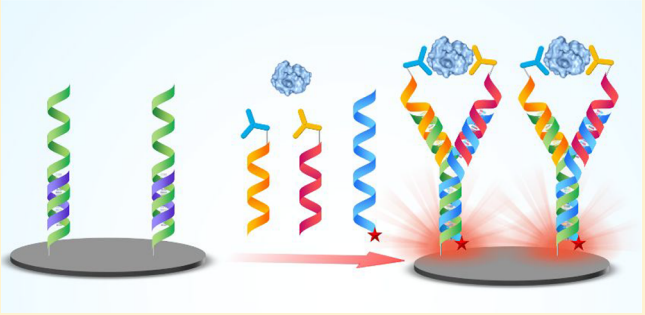
Separation-Free Electrogenerated Chemiluminescence Immunoassay Incorporating Target Assistant Proximity Hybridization and Dynamically Competitive Hybridization of a DNA Signal Probe

Bing Wang, Suwen Shi, Xiaolin Yang, Yue Wang, Honglan Qi,* Qiang Gao, and Chengxiao Zhang*

Key Laboratory of Analytical Chemistry for Life Science of Shaanxi Province, School of Chemistry and Chemical Engineering, Shaanxi Normal University, Xi'an, 710062, P.R. China

Supporting Information

ABSTRACT: A separation-free electrogenerated chemiluminescence (ECL) immunoassay for biomarkers has been developed incorporating target assistant proximity hybridization and dynamically competitive hybridization of a DNA ECL signal probe for the first time. In this work, the biomarkers of acute myocardial infarction including cardiac troponin I (cTnI), cardiac troponin T (cTnT), and myoglobin (Myo) were chosen as the model proteins while the corresponding antibody was utilized as a recognition probe and the DNA5 tagged with ruthenium complex was chosen as an ECL signal probe (DNA5-Ru1). The biosensors were fabricated by covalently coupling the capture probe DNA1 onto the surface of a glassy carbon electrode, and then, a competitor ss-DNA2 was hybridized with DNA1. When the biosensor was incubated in the solution containing a target protein, the recognition probes (DNA3-Ab1 and DNA4-Ab2), DNA5-Ru1, and the coreactant tri-*n*-propylamine, the target protein was bounded with two antibodies of the recognition probes and thus induced the sufficient proximity hybridization of DNA3 with DNA1, DNA4 with DNA5-Ru1, and DNA5-Ru1 with DNA1 and the unwinding of the competitor DNA2 with DNA1, and ECL measurement was performed in separation-free format. It was found that the hybridization base number and length of DNA1 and a competing hybridization of DNA5-Ru1 with DNA2 for DNA1 have important effects. The developed ECL method showed a quite low detection limit of 0.4 pg/mL for cTnI, 0.5 pg/mL for cTnT, and 0.5 ng/mL for Myo. The fabricated biosensor exhibited stability and reusability. This work demonstrated that the developed ECL immunoassay is a promising separation-free and flexible strategy for quantitation of multiple proteins using one biosensor.

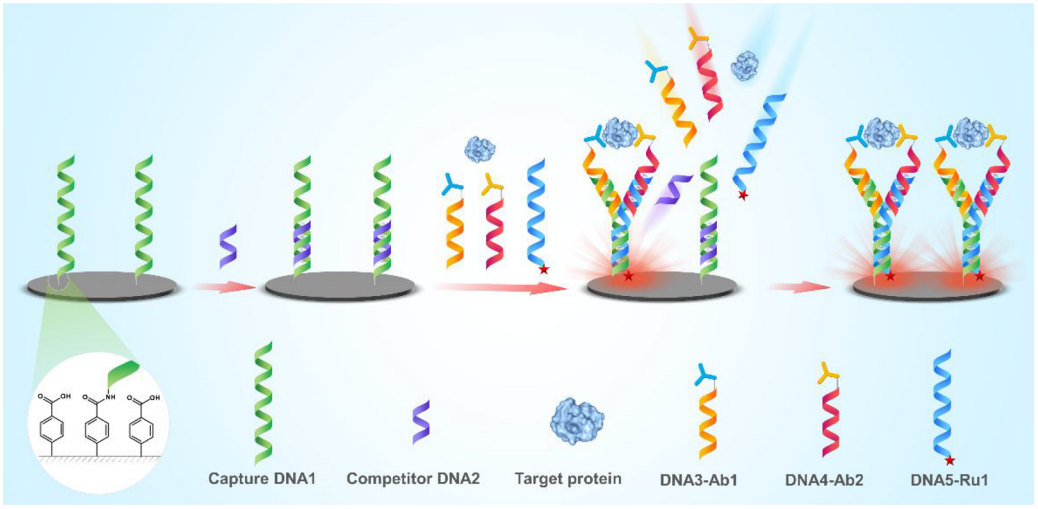


Design and development of highly sensitive, selective, and simple systems for quantifying biomarkers are of great importance in fundamental research and clinical tests and drug screening.[1−3] Immunoassay on the basis of specific binding of antigen and antibody for biomarkers (proteins) is a powerful method, since it has a high selectivity. Among the labeled immunoassays, electrogenerated chemiluminescence (ECL) immunoassay has been widely used in the clinical tests for biomarkers, since it has high sensitivity and commercially available ECL instruments, such as Elecsys[4] and Meso QuickPlex SQ 120.[5] Immunoassay can be classified as either a homogeneous (separation-free) format or a heterogeneous (separation) one. Heterogeneous formats are more straightforward to detection and also offer many advantages such as high sensitivity, high selectivity, and flexibility to the choice of detection principle and solid phase. Most reported ECL immunoassays are heterogeneous,[6−9] in which the capture probe antibody is generally immobilized on the surface of the electrodes to fabricate the biosensor and then the target protein (antigen) and the signal probe (ECL reagent-labeled antibody) are bound in sequence. However, this strategy needs several separation (washing) steps for removing the adsorptive target protein and the excess signal probe; thus, it is time-consuming and has a big accumulation error. In homogeneous ECL immunoassay (detection in a bare or chemically modified electrode), two approaches have been reported including basis of change of the diffusion coefficients between the ECL-labeled antibody and the antigen bound ECL-labeled antibody conjugate[10,11] and using magnetic beads.[12,13] The advantage on the basis of change of the diffusion coefficient format is very simple. However, its selectivity and linear range are limited. The advantage of using the magnetic beads format is high

Received: August 12, 2019
Accepted: December 6, 2019
Published: December 6, 2019

DOI: 10.1021/acs.analchem.9b03662
Anal. Chem. 2020, 92, 884−891

Scheme 1. Schematic Diagram of the Fabrication of the Biosensor and the Separation-Free ECL Immunoassay Incorporating Target Assistant Proximity Hybridization and Dynamically Competitive Hybridization of a DNA Signal Probe[a]



[a]Capture DNA1 (10 bp/50 bp for DNA5), competitor DNA2 (9 bp/9 bp for DNA1), DNA3 (20 bp/40 bp for DNA1), DNA4 (20 bp/40 bp for DNA5-Ru1), DNA5-Ru1 (10 bp/40 bp for DNA1).

Table 1. Sequences of the Oligonucleotides Used in the Experiments

strand names	sequence	bases
capture probe DNA1-H10	5′-H_2N-$(CH_2)_6$-GCA TGG TGA CAT TTT TCG TTC GTT AGG GTT CAA ATC CGC G-3′	40
capture probe DNA1-H7	5′-H_2N-$(CH_2)_6$-GCA TGG TAT TTT TCG TTC GTT AGG GTT CAA ATC CGC G-3′	37
capture probe DNA1-H5	5′-H_2N-$(CH_2)_6$-GCA TGA ATT TTC GTT CGT TAG GGT TCA AAT CCG CG-3′	35
capture probe DNA1-H10 + 5A	5′-H_2N-$(CH_2)_6$-AAA AAG CAT GGT GAC ATT TTT CGT TCG TTA GGG TTC AAA TCC GCG-3′	45
capture probe DNA1-H10 + 10A	5′-H_2N-$(CH_2)_6$-AAA AAA AAA AGC ATG GTG ACA TTT TTC GTT CGT TAG GGT TCA AAT CCG CG-3′	50
capture probe DNA1-H10 + 15A	5′-H_2N-$(CH_2)_6$-AAA AAA AAA AAA AAA GCA TGG TGA CAT TTT TCG TTC GTT AGG GTT CAA ATC CGC G-3′	55
DNA3	5′-HS-$(CH_2)_6$-CCC ACT TAA ACC TCA ATC CAC GCG GAT TTG AAC CCT AAC G-3′	40
DNA4	5′-TAG GAA AAG GAG GAG GGT GGC CCA CTT AAA CCT CAA TCC A-$(CH_2)_6$-SH-3′	40
competitor DNA2	5′-TCA CCA TGC-3′	9
DNA loop	5′-TAG GAA AAG GAG GAG GGT GGC CCA CTT AAA CCT CAA TCC ACC CAC TTA AAC CTC AAT CCA CGC GGA TTTGAA CCC TAA CG-3′	80
signal probe DNA5-Ru1	5′-CCA CCC TCC TCC TTT TCC TAT CTC TCC CTC GTC ACC ATG C-$(CH_2)_6$-Ru1-3′	40

selectivity and sensitivity. However, there exists a magnetic separation and a magnetic beads shield of the ECL emission. Therefore, a separation-free strategy for ECL immunoassay with flexible quantifying biomarkers is needed.

The proximity hybridization immunoassay (PHIA)[14] is a highly selective and more flexible assay for proteins because it uses two antibodies. A key concept in PHIA is the "proximity effect", which relies on simultaneous recognition of a target molecule by a pair of affinity probes.[15,16] A series of PHIA have been reported, such as voltammetry for carcinoembryonic antigen,[17] insulin,[18] and concanavalin A,[19] fluorescence for α-fetoprotein,[20] biotin, and digoxin,[21] fluorescence and chemiluminescence imaging method for carcinoembryonic antigen, carcinoma antigen 125, carcinoma antigen 199, and α-fetoprotein,[22] chemiluminescence for carcinoembryonic antigen,[23] polymerase chain reaction (PCR) for O-GlcNAcylation,[24] and ECL for α-fetoprotein.[25,26] Our group developed a "signal off" ECL PHIA[25] and a "signal on" ECL PHIA for α-fetoprotein.[26] However, separation-free ECL PHIA incorporating dynamically competitive hybridization has not been reported.

The aim of this work is to develop a separation-free ECL immunoassay based on target assistant proximity hybridization regulation and dynamically competitive hybridization for highly sensitive and selective detection of biomarkers. As a concept-of-proof, in this work, cardiac troponin I (cTnI) was chosen as a model analyte, since it is a protein biomarker for the screening and diagnosis of acute myocardial infarction (AMI), while two antibodies were employed as the recognition elements for one antigen and the $Ru(bpy)_3^{2+}$ derivative was chosen as an ECL signal reagent for a tagger of the ss-DNA. The assay process of the separation-free ECL immunoassay of biomarkers is depicted in Scheme 1. Five single strand DNAs (listed in Table 1) were designed as capture DNA1, competitor DNA2, ligation DNA3, ligation DNA4, and signal DNA5-Ru1.[18] The biosensor was fabricated by diazotization of an animated capture DNA1 on the surface of a glassy carbon electrode and then hybridization of a competitor DNA2. In the presence of the target protein and the coreactant tri-*n*-propylamine, the target protein was bound with two DNA-tagged antibody probes (DNA3-Ab1 and DNA4-Ab2) and then induced the hybridization of the DNA3 with DNA1 and DNA4 with DNA5, which was further hybridization of the

DOI: 10.1021/acs.analchem.9b03662
Anal. Chem. 2020, 92, 884−891

DNA1 on the biosensor. Finally, ECL measurement was directly performed in the above mixture solution. The Ab1 was different from the Ab2, and both Ab1 and Ab2 recognized different recognition sites of one antigen. The concentration of target protein was quantified by the ECL intensity. In this paper, fabrication of the biosensor and synthesis of the recognition probes (DNA3-Ab1 and DNA4-Ab2) were presented. Effects of the parameters including the length of capture DNA1, a competitor DNA2 on the ECL intensity for the DNA loop, and the dynamically competitive hybridization of the ECL signal probe DNA5-Ru1 in the ECL assay were investigated. The analytical flexibility for the ECL quantification of the target proteins including cardiac troponin I (cTnI), cardiac troponin T (cTnT), and myoglobin (Myo) biomarkers was demonstrated.

EXPERIMENTAL SECTION

Material and Apparatus. Cardiac troponin I (cTnI), cardiac troponin T (cTnT), and myoglobin (Myo) were purchased from Abcam (U.K.). Monoclonal mouse anticardiac troponin I antibodies (4T21-19C7 was termed as Ab1, 4T21-16A11 was termed as Ab2), monoclonal mouse anticardiac troponin T antibodies [4T19-1C11 was termed as Ab1 (cTnT), 4T19-9G6 was termed as Ab2 (cTnT)], and monoclonal mouse antihuman cardiac myoglobin antibodies [4M23-4E2 was termed as Ab1 (Myo), 4M23-7C3 was termed as Ab2 (Myo)] were purchased from Hytest Ltd. (Finland). Sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) and 4-aminobenzoic acid (4-ABA) were obtained from Sigma-Aldrich (USA). Dithiothreitol (DTT) was obtained from Sangon Biotechnology Co., Ltd. (Shanghai, China). Tri-*n*-propylamine (TPA), *N*-(3-(dimethylamino)-propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), and *N*-hydroxysuccinimide (NHS) were obtained from Aladdin Bio-Chem Technology Co., Ltd. (China). All oligonucleotides and signal probe DNA5-Ru1 listed in Table 1 were obtained from Sangon Biotechnology Co., Ltd. (China). Ultrapure water obtained from a Millipore water purification system (18.2 MΩ·cm, Milli-Q Reference, Millipore) was used in all assays. The recognition probes according to the references[25,27] in the Supporting Information (section 1.1 of the Supporting Information).

An HYZ-3002 ECL detector (Xi'an Heyongzhong Electronic Technology Co., Ltd., Xi'an, China) and CHI 660 E Electrochemical workstation (Chenhua, Shanghai, China) were employed in a three-electrode system consisting of the fabricated biosensor as a working electrode, a large Pt plate as an auxiliary electrode, and a commercial Ag/AgCl (saturated KCl) as a reference electrode. A UV-2450 UV−vis spectrophotometer (Shimadzu, Japan) and Bio-Rad Protein Electrophoresis and Blotting (California, USA) were employed in this work.

Fabrication of the Biosensor. The glassy carbon electrode (GCE, diameter = 2 mm) was cleaned by the protocol in a previous report.[28] Fabrication of the biosensor involved three steps including electrochemical grafting, covalent coupling, and hybridization. First was electrochemical grafting of benzoic acid on the GCE.[29,30] An 11 mg portion of $NaNO_2$ (0.16 mmol) and 27 mg of 4-aminobenzoic acid (4-ABA, 0.20 mmol) were added into 20 mL of 0.50 M hydrochloric acid and allowed to be stirred in an ice bath away from light for 5 min in order to generate the diazonium cation, and then, electrochemical grafting was performed by applying a triangular potential between +0.6 and −0.6 V vs Ag/AgCl (saturated KCl) with 50 mV/s for two cycles. After washing with ethanol and water, the grafted GCE was immersed in 80 μL of 10 mM Tris−HCl buffer (pH 8.0) containing a 1 μM capture probe (amino-terminated ss-DNA1), 10 mM NHS, and 40 mM EDC and allowed to be stirred for 1 h to covalently couple amino-terminated DNA1 with 4-aminobenzoic acid on the electrode.[31,32] Finally, hybridization of competitor DNA2 with the capture probe on the electrode was performed by immersing it into 80 μL of 500 nM competitor DNA2 for 30 min and washing with water. The fabricated biosensors were kept dry and stored at 4 °C in a refrigerator before use.

ECL Measurement of Biomarkers. For the separation-free format, the fabricated biosensor was immersed into 2.0 mL of 0.10 PBS (0.10 M Na_2HPO_4, 0.10 M NaH_2PO_4, and 0.10 M KCl, pH 7.40) containing 50 mM TPA, 20 nM DNA3-Ab1, 20 nM DNA4-Ab2, 100 nM DNA5-Ru1, and different concentrations of cTnI for 1 h at 37 °C (unless otherwise indicated). After that, a counter electrode and a reference electrode were placed into the ECL cell and the ECL measurement was performed using a linear potential scanning with 100 mV/s. ECL emission was detected by a photomultiplier tube (PMT) that was biased at −900 V. The peak ECL intensity at about +1.0 V was used to quantify the concentration of the target protein.

RESULTS AND DISCUSSION

Characterization of the Recognition Probes and the Biosensor. The recognition probes were synthesized by using three targets corresponding to six specific antibodies and two thiolated DNA strands (DNA3 and DNA4) on the basis of a coupling reaction between the amino group of the antibody and the sulfydryl group of thiolated DNA using heterobifunctional sulfo-SMCC as a linker according to refs 25 and 27 with some modifications (section 1.1 and Figure S1A of the Supporting Information). As a representative example, the recognition probe DNA3-Ab1 synthesized was characterized using UV−vis adsorption spectrophotometry (Figure S1B of the Supporting Information). Clearly, the absorption spectrum of DNA3-Ab1 showed a merged peak at 262 nm compared with a typical peak of the DNA3 at 260 nm and a typical peak of the antibody (Ab1) at 280 nm, indicating that ss-DNA3 was successfully coupled on Ab1.[33] On the basis of the absorbance ratio (260 nm vs 280 nm), the molecular ratio of ss-DNA3 to Ab1 was calculated to be 9.4:1, on the basis that the concentration of Ab1 in DNA3-Ab1 solution was calculated to be 86 nM (dilution 1:8) (section 2.1 of the Supporting Information).[34] The concentration of DNA3-Ab1 in the store solution was expressed as 0.69 μM.

In order to develop a reusable biosensor for the separation-free ECL immunoassay, the biosensor was fabricated using a double covalent coupling method for assembly of the capture probe on the surface of GCE. Our group has employed a double covalent coupling method for the fabrication of highly sensitive and reusable ECL biosensors by electrochemical grafting using 4-aminobenzenesulfonic acid[28] and 4-aminobenzoic acid.[31] In this work, we employed an electrochemical grafting using 4-ABA. This design benefits a direct contact of a benzoic group to the surface of the GC electrode for the biosensor. Fabrication of the biosensor was performed in three steps including electrochemical grafting and covalent attachment of the amino-terminated capture DNA1 to the carboxylic

DOI: 10.1021/acs.analchem.9b03662
Anal. Chem. 2020, 92, 884−891

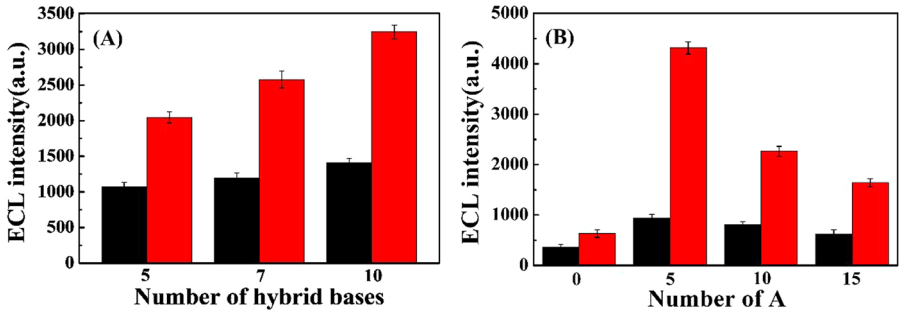


Figure 1. (A) Dependence of the ECL intensity on the hybridization numbers of capture probe DNA1. (B) Dependence of the different poly-A bases of capture probe DNA1-10H on the ECL intensity. The capture probe was 1 μM, the loop was 50 nM, and the signal probe DNA5-Ru1 was 500 nM. The black column was in the absence of the loop, and the red column was in the presence of the loop. Measurement solution: 50 mM TPA−0.1 M PBS (pH 7.4). Scan rate: 0.1 V/s.

acid-terminated modified GCE and hybridization of a competitor DNA2. In the first step, GCE by introduction of 4-ABA was achieved by electrochemical reduction of the diazonium cation generated in situ from 4-ABA (Figure S2A of the Supporting Information). The cyclic voltammograms obtained in the electrochemical grafting process (Figure S2B of the Supporting Information) showed that one reduction peak centered at −0.15 V in the negative scan of first cycle, corresponding to the reduction of the aryl diazonium salt while the redox peak disappears in the negative scan of the second cycle, indicating that two negative scans is enough to form a carboxylic acid layer on the surface of the electrode. The scan for two cycles was employed to obtain a stable layer.

The biosensor fabricated was characterized by electrochemical impedance spectroscopy.[35] The result indicates that the biosensor is successfully fabricated and possibly binds with target protein cTnI, DNA3-Ab1, DNA4-Ab2, and ECL signal probe DNA5-Ru1 (section 2.2 and Figure S3 of the Supporting Information).

Effect of the Hybridization Number and Length of Capture Probe DNA1. The previous report[36] and our preliminary experiment results showed that the ECL behaviors of the fabricated biosensors for the DNA loop (sequence shown in Table 1), which was employed to mimic the function of the antibodies and target protein, were the same as those for DNA3-Ab1, DNA4-Ab2, and target protein. Therefore, the recognition probes (DNA3-Ab1 and DNA4-Ab2) and the target protein were replaced by the DNA loop in this study (section 1.2 of the Supporting Information).

In order to study the effect of the hybridization number of the capture probe DNA1 with DNA5-Ru1 on the ECL intensity, the DNA1 sequences used were designed by partly deleting the hybridization bases of DNA1 sequences reported in ref 18. Considering that the hybridization number of the capture probe DNA1 with DNA5-Ru1 is more important, the competitor DNA2 was not employed for the fabrication of the biosensor in this stage. Figure S4 (Supporting Information) shows the ECL intensity−potential profiles on different biosensors fabricated by the capture probe DNA1 with different hybridization numbers, and Figure 1A shows the dependence of the ECL intensity on the hybridization numbers of capture probe DNA1. It can been seen that the ECL intensity directly increases with an increase of the hybridization numbers of capture probe DNA1 from 5 to 10, while the increase degree of the ECL intensity in the presence of the loop is bigger than that in the absence of the loop. These observations are attributed to the fact that longer hybridization numbers in the capture probe DNA1 lead to higher proximity ligation efficiency.[18]

Additionally, polyacrylamide gel electrophoresis (PAGE) analysis[37] was performed to make an insight into the hybridizations between DNA1-H10 and ECL signal probe DNA5-Ru1 and among DNA1-H10, the DNA loop, and DNA5-Ru1 (section 1.4 of the Supporting Information). The PAGE images (Figure S5 of the Supporting Information) showed that, compared with DNA ladder markers (lane 1, 25−500 bases), three clear bands including lane 2 (DNA1, ∼35 bp), lane 3 (DNA5-Ru1, ∼37 bp), and lane 4 (DNA loop, 75−150 bp) were observed to be partly matched with DNA1- H10 (40 bp), DNA5-Ru1 (higher than 40 bp), and the DNA loop (80 bp), respectively. For the DNA loop, the observed value was higher than the real value, attributed to dimerization of the DNA loop. Lane 5 showed the observed band at about 40 bp, suggesting that no obvious hybridization between DNA1 and DNA5-Ru1 occurred in the presence of the DNA loop. Lane 6 showed the observed bands appear at 500−150 bp, which is much higher than the predicted value from a simple summation of DNA1 and DNA5-Ru1 and the DNA loop. These observations partly suggest that the hybridizations can be performed among DNA1-H10 and the DNA loop and DNA5-Ru1. This was also supported by the predicated changes of Gibbs free energy at 25 °C (ΔG_{25}) (Figure S6 of the Supporting Information).

For the hybridization of the capture probe with DNA5, the change of the Gibbs free energy of the capture probe DNA1-H10 (ΔG_{25} = −13.6 kcal/mol) is much smaller than that of the capture probe DNA1-H7 (ΔG_{25} = −10.0 kcal/mol) and the capture probe DNA1-H5 (ΔG_{25} = −7.5 kcal/mol). This indicates that the hybridization efficiency of DNA1-H10 is higher than that of DNA1-H7 and DNA1-H5 for DNA5-Ru1. Therefore, DNA1-H10 was employed as a capture probe in this work.

Additionally, the effect of the length of the capture probe DNA1-H10 on the ECL intensity was also studied by adding different lengths of poly-A base into the capture probe DNA1-H10. The designed capture probes DNA1-H10 make different distances from the signal probe DNA5-Ru1 to the surface of the GCE but keep the same hybridization number of the capture probe DNA1-H10 with the signal probe DNA5-Ru1. A series of the biosensors were fabricated using different DNA1-H10 capture probes, which were designed by the addition of different poly-A bases (0, 5, 10, 15) in between alkyl and the originated capture probe DNA1-H10 (40 bases). Figure 1B and Figure S7 (Supporting Information) show the ECL

DOI: 10.1021/acs.analchem.9b03662
Anal. Chem. 2020, 92, 884−891

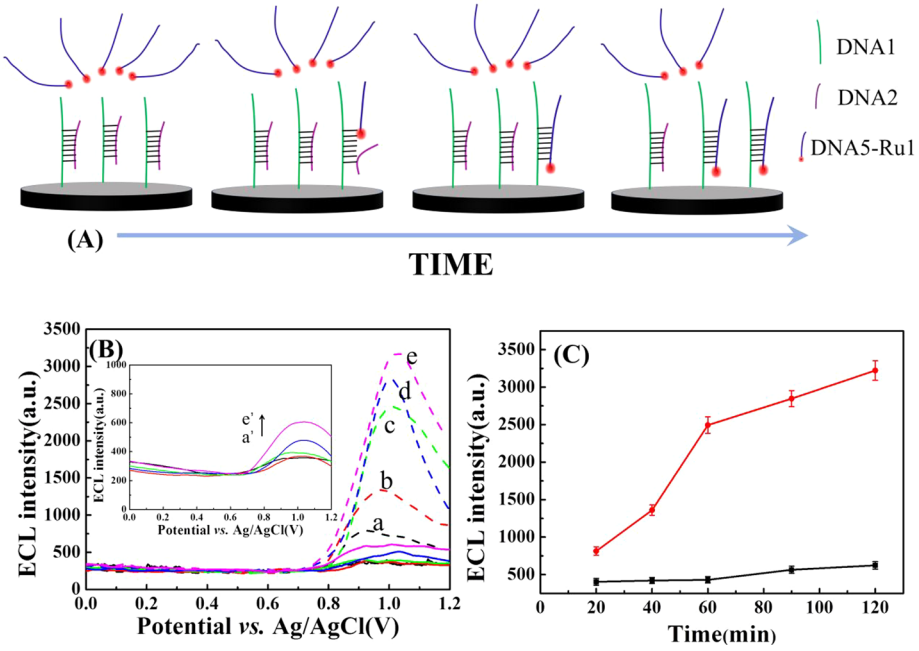


Figure 2. (A) Depiction of a dynamically competitive hybridization of the signal probe (DNA5-Ru1) with competitor DNA2 for the capture probe DNA1. (B) ECL intensity−potential profiles of the biosensors with competitor DNA2 at different incubation times in the presence of other probes and targets (20 nM DNA3-Ab1, 0.6 ng/mL cTnI, 20 nM DNA4-Ab2, 1 μM signal DNA5-Ru1) and 50 mM TPA in 0.10 M PBS (pH 7.40). Scan rate: 0.1 V/s. (C) Dependence of the ECL intensity on the incubation time, in the presence of cTnI (red line) and in the absence of cTnI (black line).

intensity−potential profiles of the fabricated biosensors using the capture probe DNA1-H10 for the DNA loop and the dependence of the ECL intensity on the different poly-A bases. It was found that the maximum ECL intensity occurred at five poly-A bases in the capture probe DNA1-H10 in the presence/absence of the DNA loop and the ECL intensity in the presence of the DNA loop was higher than that in the absence of the DNA loop. These observations are attributed to the fact that the capture probe DNA1-H10 containing five poly-A bases makes a suitable distance of the signal probe DNA5-Ru1 close to the surface of the GCE and leads to a higher proximity ligation efficiency.[38] Therefore, the capture probe DNA1-H10 + 5A (Table 1) was employed as the capture probe in the following experiments.

Dynamically Competitive Hybridization of the Signal Probe with the Competitor DNA2 for the Capture Probe. In order to check whether the competitor DNA2 can reduce the ECL background in the proposed strategy, the ECL intensity was examined in the presence/absence of the competitor ss-DNA2 using the DNA loop. The experiment was performed by following the protocol as described in the Experimental Section (section 1.2 of the Supporting Information) except that the DNA loop replaced the recognition probes and target protein. The obtained result (Figure S8 of the Supporting Information) showed that the ECL peak intensity of the biosensor fabricated by hybridization without the competitor DNA2 in the presence of the DNA loop (770 au) was higher than that in the absence of the DNA loop (423 au), while the ECL peak intensity of the biosensor fabricated by hybridization with the competitor DNA2 in the presence of the DNA loop (634 au) was higher than that in the absence of the DNA loop (360 au). These observations are attributed to the fact that the hybridization of the competitor DNA2 with the capture probe DNA1-H10 on the electrode obstructs an efficient hybridization of the signal probe DNA5-Ru1 with the capture probe DNA1-H10.[18] This indicates that the competitor DNA2 can slightly reduce the ECL background in the proposed strategy.

Importantly, the feasibility of the proposed separation-free approach was examined using cTnI as a representative target in the time course. Figure 2A shows a depiction of a dynamically competitive hybridization of the signal probe (DNA5-Ru1) with competitor DNA2 for the capture probe DNA1 so that the proposed approach is easily understood. Figure 2B shows ECL intensity−potential profiles, while Figure 2C shows the dependence of the ECL intensity on the incubation time. In the absence of cTnI, the ECL intensity nearly keeps a constant value in the incubation time from 20 to 60 min, attributed to a constant signal probe DNA5-Ru1 in the testing solution, and then quite slowly increases from 431 to 624 au with increasing incubation time from 60 to 120 min, attributed to a contribution of the signal probe DNA5-Ru1 adsorbed on the surface of the electrode. In the presence of cTnI, the ECL intensity sharply increases from 814 to 2494 au with increasing incubation time from 20 to 60 min, attributed to the fact that the target assistant proximity hybridization induced the hybridization of the signal probe DNA5-Ru1 with the capture probe DNA1, which led to the Ru1 of DNA5-Ru1 being close to the surface of the electrode and makes a high ECL intensity compared with the ECL emission produced through the diffusion of DNA5-Ru1 from the solution. In the presence of cTnI, the ECL intensity then slowly increases from 2494 to 3221 au with increasing incubation time from 60 to 120 min. This is attributed to the fact the increased amount of the signal probe DNA5-Ru1 through the adsorption on the surface of the electrode and the hybridization with DNA1 is relatively low compared with that before 60 min. When the incubation time is fixed at 60 min, the increased ECL intensity can be

DOI: 10.1021/acs.analchem.9b03662
Anal. Chem. 2020, 92, 884−891

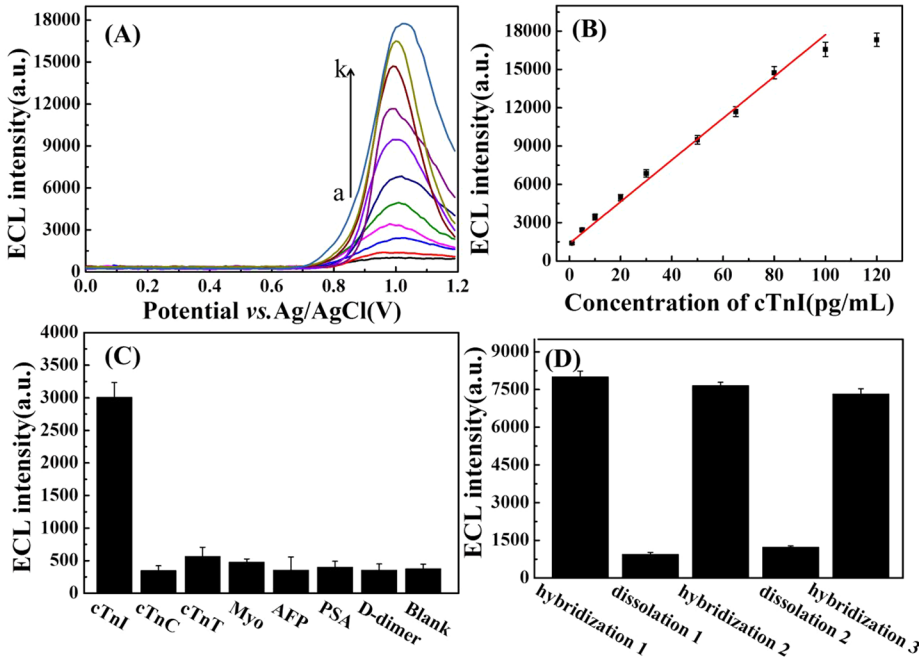


Figure 3. (A) ECL intensity−potential profiles and (B) calibration curve for cTnI. (a) 0 pg/mL, (b) 1 pg/mL, (c) 5 pg/mL, (d) 10 pg/mL, (e) 20 pg/mL, (f) 30 pg/mL, (g) 50 pg/mL, (h) 65 pg/mL, (i) 80 pg/mL, (g) 100 pg/mL, (k) 120 pg/mL. (C) ECL response of the biosensor for the detection of 10 pg/mL cTnI, 0.5 ng/mL cTnC, 0.2 ng/mL cTnT, 100 ng/mL Myo, 25 ng/mL AFP, 5.0 ng/mL PSA, or 200 ng/mL D-dimer. (D) Regeneration of the biosensor in the presence of other probes and 40 pg/mL cTnI.

employed to qualify the target cTnI. These results indicate that the proposed separation-free strategy on the basis of the dynamically competitive hybridization of the ECL signal probe for the competitor DNA2 is feasible for the determination of biomarker cTnI. It should be noted that the specific role of the competitor ss-DNA2 in the assay except for reducing the background signal is to compete with the DNA5 in the signal probe (DNA5-Ru1) for the capture DNA1. Therefore, this dynamically competitive hybridization of the signal probe (DNA5-Ru1) plays an important role in the separation-free ECL immunoassay proposed in this work. Although without a toe-hold recognition site in DNA1, the DNA5-Ru1 can hybridize with DNA1 in the presence of DNA2. This is supported by the change of the Gibbs free energy of the hybridization between the capture probe DNA1-H10 and DNA5 (ΔG_{25} = −13.6 kcal/mol) and between the capture probe DNA1-H10 and DNA2 (ΔG_{25} = −11.3 kcal/mol) (Figure S6 of the Supporting Information). In order to obtain a relatively high sensitivity in a short time, therefore, an incubation time of 60 min was chosen in the determination of biomarkers. In summary, in a fixed incubation time, introduction of the competitor DNA2 and the signal probe DNA5-Ru1 in this design made the proposed separation-free strategy feasible.

Analytical Performance for cTnI. The analytical performances of the proposed separation-free ECL immunoassay for the detection of cTnI were examined under the optimized conditions. Figure 3A,B shows the representative ECL intensity−potential profiles and calibration curves for the determination of cTnI. The ECL intensity increases with increasing concentration of cTnI in the range from 1 to 100 pg/mL for cTnI, and the linear regression equation is I = 163.4C + 1373 (C units in pg/mL, R^2 = 0.9936). The limit of detection of cTnI was calculated to be 0.4 pg/mL (3σ), which is comparable with that (0.79 pg/mL cTnI) reported using a heterogeneous ECL biosensor.[39] More information on the comparison of the linear range and detection limit reported method with that of the developed method was provided (Table S1 of the Supporting Information). The cutoff value of cTnI in the clinical test is 0.3 ng/mL.[40] Therefore, a highly sensitivity of the developed method is evident.

The selectivity of the developed ECL immunoassay for cTnI (10 pg/mL) as a representative analyte using the fabricated biosensors was examined by recording the ECL intensity in 0.1 M PBS containing 50 mM TPA, 50 nM DNA3-Ab1, 50 nM DNA4-Ab2, 100 nM DNA5-Ru1, and one biomarker. The tested biomarkers included cTnI (50 pg/mL), cTnC (0.5 ng/mL), cTnT (0.2 ng/mL), Myo (100 ng/mL), AFP (25 ng/mL), PSA (5.0 ng/mL), and D-dimer (200 ng/mL). The concentration of the tested biomarkers was controlled to be 2-fold or higher than that of the critical values. A remarkable change in the ECL intensity was only observed for 10 pg/mL cTnI, while negligible changes in the ECL intensity were observed in the presence of other tested biomarkers (Figure 3C). This result indicates that the developed ECL assay has a high selectivity for cTnI (40 pg/mL) as a representative analyte.

To demonstrate the multiple uses of the biosensor in the proposed approach, one fabricated biosensor was applied to multiple-detect cTnI (40 pg/mL). After the first use of the biosensor for detecting cTnI (40 pg/mL) in the process described in the Experimental Section, the used biosensor was retrieved and treated by putting it into 95 °C water for 5 min to make DNA unwinding and by washing with water. The treated biosensor was rehybridized with DNA2. The rehybridized biosensor was put into the mixture solution containing 50 nM DNA3-Ab1, 50 nM DNA4-Ab2, 100 nM DNA5-Ru1, and 50 pg/mL cTnI. The result (Figure 3D) showed that the biosensor can be generated for least three times. This indicates that the capture probe assembled on the

DOI: 10.1021/acs.analchem.9b03662
Anal. Chem. 2020, 92, 884−891

surface of GCE by using the double covalent coupling method is stable.

It is well-known that the most challenging issue in separation-free assays is the sometimes deleterious effect of a complex matrix. Therefore, the potential applicability of the developed method was examined by assaying cTnI in six human serum samples. The human serum samples were provided by Shaanxi Provincial Peoples Hospital, and the analytical results for cTnI human serum samples were obtained by using the chemiluminescent (CL) immunoassay method. In the developed ECL immunoassay, interruptions from other macromolecules in serum were eliminated by mainly depending on the specificity of the antibody used. Other macromolecules in serum could not induce the proximity hybridization and scarcely adsorbed on the surface of the electrode, since the surface of the electrode was grafted by aminobenzoic acid. Table S2 (Supporting Information) lists the analytical results for cTnI human serum samples from our method. There is no significant difference using the t test with a confidence level of 95%.[41] This indicates that the developed method can be applied in real applicability as a simple and selective method for biomarker detection. This is attributed to the target assistant proximity hybridization and dynamically competitive hybridization of the DNA ECL signal probe.

Flexibility for cTnT and Myo Biomarkers. In order to demonstrate the flexible strategy of the proposed approach for specific protein quantitation, the fabricated biosensors were extended for ECL quantitation of AMI biomarkers, Myo and cTnT, in which the recognition probes were only changed while the signal probe DNA5-Ru1 was the same. Here, DNA3-Ab1 (cTnT) and DNA4-Ab2 (cTnT) were used for cTnT, while DNA3-Ab1 (Myo) and DNA4-Ab2 (Myo) were used for Myo. As shown in Figure S9A, the ECL intensity is directly proportional to the concentration of cTnT in the range from 1 to 120 pg/mL, and the linear regression equation is I = 1128.9C + 1415 (C units in pg/mL, R^2 = 0.9915). The detection limit of cTnT was calculated to be 0.5 pg/mL (3σ). As shown in Figure S9B, the ECL intensity is directly proportional to the concentration of Myo in the range from 1 to 10 ng/mL, and the linear regression equation is I = 77.2C + 1159.6 (C units in ng/mL, R^2 = 0.9973). The detection limit of Myo was calculated to be 0.5 ng/mL (3σ). The cutoff value in the clinical test is 0.2 ng/mL for cTnT[42] and 20 ng/mL for Myo.[43] It should be noticed that, in the developed strategy, the sensitivity and detection limit for the three targets cTnI, cTnT, and Myo are different. This is maybe explained by the following reasons: (1) Different antibodies have different binding constants for different antigens. (2) The concentrations of the recognition probes DNA3-Ab1 and DNA4-Ab2 were not all the same in the experiments. (3) The molecular ratio of ss-DNA3 to Ab1 (or ss-DNA4 to Ab2) was not actually the same. Additionally, the low detection limits for cTnT and Myo are obtained (Table S1 of the Supporting Information). Importantly, the results demonstrated that the proposed strategy is a promising flexible strategy for specific protein quantitation using one fabricated biosensor.

CONCLUSION

A flexible and separation-free ECL immunoassay incorporating target assistant proximity hybridization and dynamically competitive hybridization of the ss-DNA ECL signal probe was developed for the first time. A biosensor was designed to be fabricated by covalently coupling a capture probe amino-terminated DNA1 with 4-ABA on GCE and then primarily hybridizing a competitor ss-DNA2. When the biosensor was simply incubated in the mixture solution containing target protein, the recognition probes, ECL signal probe, and TPA for 1 h, ECL measurement was directly performed. It was found that the hybridization base number and length of the capture probe DNA1 have important effects on the ECL intensity in the designed system, since the maximum ECL intensity was achieved by addition of 5 A bases into the capture DNA1 and the optimal capture DNA1 containing 10 bases for the hybridization with the ECL signal probe DNA5-Ru1. The fabricated biosensor was stable and reusable. This work demonstrated that the separation-free ECL immunoassay can be applied for assay of real serum samples with satisfactory results and is a flexible strategy for specific protein (cTnI, cTnT, Myo) quantitation using one fabricated biosensor. The proposed strategy is amenable to highly sensitive and selective quantitation of the protein biomarkers in a separation-free format and will be used for a wide variety of target proteins.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.9b03662.

Experiments for synthesis of the DNA−Ab conjugates, ECL measurements and PAGE analysis; results for UV−vis absorption spectra, effect of the hybridization numbers, impedance spectra, PAGE analysis, predicted hybridizations, calibration curves of cTnT and Myo, and analytical results of cTnI in human serum samples (PDF)

AUTHOR INFORMATION

Corresponding Authors

*E-mail: cxzhang@snnu.edu.cn. Phone: +86-29-8153 0833. Fax: +86-29-8153 0833.

*E-mail: honglanqi@snnu.edu.cn. Phone: +86-29-8153 0833. Fax: +86-29-8153 0833.

ORCID

Bing Wang: 0000-0002-8981-6721

Honglan Qi: 0000-0002-2268-0073

Qiang Gao: 0000-0002-0775-3414

Chengxiao Zhang: 0000-0003-2829-5122

Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Financial support from the National Natural Science Foundation of China (Nos. 21775098 and 21775097) and the Fundamental Research Funds for the Central Universities (No. GK201801006) is gratefully acknowledged.

REFERENCES

(1) Philp, R. P.; Oung, J. N. *Anal. Chem.* **1988**, *60*, 887A−896A.

(2) Luo, X.; Davis, J. J. *Chem. Soc. Rev.* **2013**, *42*, 5944−5962.

(3) Rasooly, A.; Jacobson, J. *Biosens. Bioelectron.* **2006**, *21*, 1851−1858.

(4) Miao, W. *Chem. Rev.* **2008**, *108*, 2506−2553.

DOI: 10.1021/acs.analchem.9b03662
Anal. Chem. 2020, 92, 884−891

(5) Schulz, A. R.; Mälzer, J. N.; Domingo, C.; Jürchott, K.; Grützkau, A.; Babel, N.; Nienen, M.; Jelinek, T.; Niedrig, M.; Thiel, A. *J. Immunol.* **2015**, *195*, 4699−4711.
(6) Lin, Y.; Jia, J.; Yang, R.; Chen, D.; Wang, J.; Luo, F.; Guo, L.; Qiu, B.; Lin, Z. *Anal. Chem.* **2019**, *91*, 3717−3724.
(7) Liang, G.; Liu, S.; Zou, G.; Zhang, X. *Anal. Chem.* **2012**, *84*, 10645−10649.
(8) Miao, W.; Bard, A. J. *Anal. Chem.* **2004**, *76* (23), 7109−7113.
(9) Zhan, W.; Bard, A. J. *Anal. Chem.* **2007**, *79* (2), 459−463.
(10) Tanaka, M.; Kamiya, S.; Shibue, A.; Namba, K.; Aizawa, M. *Sens. Actuators, B* **1993**, *13*, 184−187.
(11) Qi, H.; Zhang, C. *Anal. Chim. Acta* **2004**, *501*, 31−35.
(12) Zhang, J.; Qi, H.; Li, Z.; Zhang, N.; Gao, Q.; Zhang, C. *Anal. Chem.* **2015**, *87*, 6510−6515.
(13) Wei, Y.; Wang, Y.; Wang, J.; Yang, X.; Qi, H.; Gao, Q.; Zhang, C. *Anal. Bioanal. Chem.* **2019**, *411*, 4203−4211.
(14) Fredriksson, S.; Gullberg, M.; Jarvius, J.; Olsson, C.; Pietras, K.; Gústafsdóttir, S. M.; Östman, A.; Landegren, U. *Nat. Biotechnol.* **2002**, *20*, 473−477.
(15) Zhang, H.; Li, F.; Dever, B.; Wang, C.; Li, X.-F.; Le, X. C. *Angew. Chem., Int. Ed.* **2013**, *52*, 10698−10705.
(16) Gullberg, M.; Gustafsdottir, S. M.; Schallmeiner, E.; Jarvius, J.; Bjarnegard, M.; Betsholtz, C.; Landegren, U.; Fredriksson, S. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 8420−8424.
(17) Ren, K.; Wu, J.; Ju, H.; Yan, F. *Anal. Chem.* **2015**, *87*, 1694−1700.
(18) Hu, J.; Wang, T.; Kim, J.; Shannon, C.; Easley, C. J. *J. Am. Chem. Soc.* **2012**, *134*, 7066−7072.
(19) Liu, B.; Zhang, B.; Chen, G.; Yang, H.; Tang, D. *Anal. Chem.* **2014**, *86*, 7773−7781.
(20) Zou, M.; Li, D.; Yuan, R.; Xiang, Y. *Biosens. Bioelectron.* **2017**, *92*, 624−629.
(21) Yan, X.; Le, X. C.; Zhang, H. *Anal. Chem.* **2018**, *90*, 9667−9672.
(22) Zong, C.; Wu, J.; Liu, M.; Yan, F.; Ju, H. *Chem. Sci.* **2015**, *6*, 2602−2607.
(23) Zong, C.; Wu, J.; Liu, M.; Yang, L.; Liu, L.; Yan, F.; Ju, H. *Anal. Chem.* **2014**, *86*, 5573−5578.
(24) Robinson, P. V.; Tsai, C.; Groot, A. E.; McKechnie, J. L.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2016**, *138*, 10722−10725.
(25) Gao, H.; Wang, X.; Li, M.; Qi, H.; Gao, Q.; Zhang, C. *Biosens. Bioelectron.* **2017**, *98*, 62−67.
(26) Wang, X.; Gao, H.; Qi, H.; Gao, Q.; Zhang, C. *Anal. Chem.* **2018**, *90*, 3013−3018.
(27) Zong, C.; Wu, J.; Liu, M.; Yang, L.; Yan, F.; Ju, H. *Anal. Chem.* **2014**, *86*, 9939−9944.
(28) Sun, B.; Qi, H.; Ma, F.; Gao, Q.; Zhang, C.; Miao, W. *Anal. Chem.* **2010**, *82*, 5046−5052.
(29) Baranton, S.; Belanger, D. *J. Phys. Chem. B* **2005**, *109*, 24401−24410.
(30) Boland, S.; Barriere, F.; Leech, D. *Langmuir* **2008**, *24*, 6351−6358.
(31) Ma, F.; Sun, B.; Qi, H.; Zhang, H.; Gao, Q.; Zhang, C. *Anal. Chim. Acta* **2011**, *683*, 234−241.
(32) Liu, J.; Gooding, J. J.; Paddon-Row, M. N. *Chem. Commun.* **2005**, 631−633.
(33) Zhou, Z.; Xiang, Y.; Tong, A.; Lu, Y. *Anal. Chem.* **2014**, *86*, 3869−3875.
(34) Ren, K.; Wu, J.; Zhang, Y.; Yan, F.; Ju, H. *Anal. Chem.* **2014**, *86*, 7494−7499.
(35) Wang, B.; Jing, R.; Qi, H.; Gao, Q.; Zhang, C. *J. Electroanal. Chem.* **2016**, *781*, 212−217.
(36) Kim, J.; Hu, J.; Sollie, R. S.; Easley, C. J. *Anal. Chem.* **2010**, *82*, 6976−6982.
(37) Laemmli, U. K. *Nature* **1970**, *227*, 680−685.
(38) Pittman, T. L.; Miao, W. *J. Phys. Chem. C* **2008**, *112*, 16999−17004.
(39) Yang, X.; Zhao, Y.; Sun, L.; Qi, H.; Gao, Q.; Zhang, C. *Sens. Actuators, B* **2018**, *257*, 60−67.
(40) Mehta, N. J.; Jani, K.; Khan, I. *Am. Heart J.* **2003**, *145*, 821−825.
(41) Amabile, T. *Confidence intervals, consortium for mathematics and its applications (U.S.)*; Chedd-Angier Production Company: 1989.
(42) Ottani, F.; Galvani, M.; Nicolini, F. A.; Ferrini, D.; Pozzati, A.; Pasquale, G. D.; Jaffe, A. S. *Am. Heart J.* **2000**, *140*, 917−927.
(43) Woo, J.; Lacbawan, F. L.; Sunheimer, R.; Lefever, D.; Mccabe, J. B. *Am. J. Clin. Pathol.* **1995**, *103*, 725−729.

DOI: 10.1021/acs.analchem.9b03662
Anal. Chem. 2020, 92, 884−891