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Multiple chemiluminescence immunoassay detection of the concentration ratio of glycosylated hemoglobin A1c to total hemoglobin in whole blood samples

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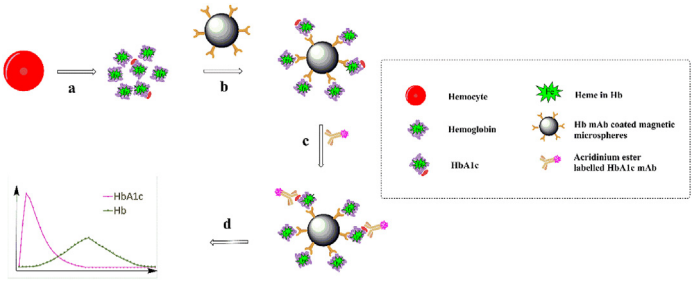
HIGHLIGHTS

- A novel time-resolved chemiluminescence assay approach for HbA1c/Hb detection was reported for the first time.
- The chemiluminescence peaks of acridine ester and heme were distinguished to determine the concentrations of HbA1c/Hb.
- This assay provides apparent advantages with excellent reproducibility and accuracy compared with the only HbA1c method.

GRAPHICAL ABSTRACT



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ABSTRACT

The concentration ratio of glycosylated hemoglobin A1c (HbA1c) to total hemoglobin (Hb) has long been used to accurately determine stagewise diabetes because this parameter represents a reliable and accurate biomarker of mean 90-day blood glucose values. In this paper, we report a time-resolved chemiluminescence assay that can detect both Hb and HbA1c. For the determination of Hb, the interaction of heme in Hb with H_2O_2 in NaOH solution was performed to generate a chemiluminescence peak. HbA1c was detected using a sandwich immunoassay based on an acridine ester-labeling method using the same Hb chemiluminescence trigger system. The results showed that the repeatability %CV of the proposed method for multiple detections of HbA1c and Hb ranged from 1.22 to 2.21%, with a median value of 1.73%, while the within-site reproducibility %CV ranged from 2.13 to 3.27%, with a median value of 2.81%. Compared with the conventional HPLC method (BIO-RAD D10 system), the correlation coefficient was 0.9959. In conclusion, a time-resolved multiple chemiluminescence immunoassay biosensor

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for HbA1c/Hb detection was established, and the method has excellent reproducibility and accuracy, thus demonstrating great potential for clinical application.

1. Introduction

Chronic hyperglycemia of diabetes is a condition in which blood glucose levels are elevated because of shortcomings in the production of insulin, which leads to lasting damage, dysfunction, and organ failure [1]. These complications ultimately cause significant mortality and morbidity in patients with diabetes [2]. The increase in blood glucose concentrations results in increases in the proportion of HbA1c; thus, the percentage of HbA1c in the blood can act as a biomarker of average blood glucose concentration over the past three months [3,4]. HbA1c is an extremely important hemoglobin derivative formed by nonenzymatic posttranslational modification of the N-terminal valine of the b-chains of Hb [5], and it has become an established parameter in the management of diabetes mellitus in terms of both diagnosis and monitoring [6–8]. Methods for measuring HbA1c are based on a variety of analytical principles, such as separation based on charge (ion-exchange chromatography in HPLC or mini-columns, electrophoresis) [9–11], affinity binding of HbA1c (affinity chromatography) [12,13], or immunoassays [14–16]. HPLC is considered the gold standard for HbA1c analysis among the various methods because it can detect both Hb and HbA1c concentrations accurately in whole blood to obtain the HbA1c percentage [4,8,17,18]. However, the HPLC method is generally considered to be expensive, and complicated, time-consuming, and it is easily affected by interfering components and environmentally unfriendly due to the use of hazardous organic solvents [4,11,17]. Many previous commercial immunoassays have been designed to measure the absolute amount of HbA1c without providing percentage information [13,19,20]. The absolute amount of HbA1c determined by only the HbA1c immunoassay may lead to misinterpretations of the test results and misjudgments of the clinical severity. Therefore, it is necessary to develop an alternative immunoassay method for the simultaneous detection of HbA1c and Hb [14,21]. Multiplexed immunoassays in a single run are gaining increasing attention due to their high-throughput advantage in the clinical diagnosis area. One of the strategies is the spatial-resolved method, which utilizes a universal probe to label all analytes, and another is the time-resolved method, which applies multiple probes because the signals from different probes can be easily distinguished by various parameters, such as wavelength. In time-resolved chemiluminescence analysis, some reactions are fast (flash type) and produce relatively short but intense signals (seconds), some reactions are slow (glow type) and provide a longer lifetime of emissions (minutes to hours), and some reactions are oscillatory and present chemiluminescent emissions that periodically increase and decay. These chemiluminescence systems with distinct kinetic characteristics provide possible pathways to detect multiple analytes in a single run using a time-resolution strategy [22–25]. Based on long-term research by our team in the field of chemiluminescent microparticle immunoassay [26,27], we developed a time-resolved multiple chemiluminescence assay for the concentration ratio of HbA1c to total Hb in whole blood samples. A schematic illustration is shown in Fig. 1. Erythrocytes are lysed to release Hb and HbA1c after sample preparation, magnetic nanoparticles coated with anti-Hb antibodies selectively capture the total Hb, and anti-HbA1c antibodies labeled with acridinium ester recognize HbA1c to form an immunocomplex. After washing, the heme in Hb and acridinium ester indirectly labeled on HbA1c simultaneously react with H_2O_2 in alkaline solution to generate chemiluminescence. The height and time of the chemiluminescence peaks of acridine ester and iron porphyrin can be distinguished by optimizing the surfactant. Finally, the simultaneous determination of the concentration of total HbA1c and Hb was realized.

2. Materials and methods

2.1. Materials

2′,6′-Dimethylcarbonylphenyl-10-sulfopropylacridinium-9-carboxylate4′-NHS ester (NSP-DMAE-NHS) was purchased from Shanghai Materwin (China). Tween-20, N-hexadecyltrimethylammonium chloride (CTAC), dimethylsulfoxide (DMSO) 30% w/v hydrogen peroxide solution, sodium hydroxide, and 70% w/v nitric acid were purchased from Aladdin Reagent (China). Sulfuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC) was purchased from Thermo Fisher (USA), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), Triton X-100, bovine serum albumin (BSA), and N-hydroxysuccinimide (NHS) were purchased from Sigma–Aldrich (USA). The Sephadex G-25 desalting column was purchased from GE Healthcare Life Sciences (USA). Carboxyl magnetic nanoparticles were purchased from JSR Life Sciences (Japan). Polyclonal goat anti-Hb antibody was purchased from Fitzgerald Industries International (USA). Two purified protein materials (human HbA1c and Hb) were prepared by Getein Biotechnology (China).

2.2. Preparation of anti-HbA1c antibody

The anti-HbA1c antibody was developed by immunizing rabbits with a synthetic peptide corresponding to the N-terminal valine of Hb beta-chains, and the sequence of the synthesized HbA1c fragment is as follows: D-Glucose-VHLTPE-C. Moreover, cysteine modified on amino acid sequences can be used for biological coupling. A general procedure that has been used to attach BSA to the synthetic peptide via SMCC consists of the following steps: to prepare the solution of the synthetic peptide in the phosphate buffer, SMCC is dissolved in DMSO, and BSA is added to the phosphate buffer. Under stirring, the peptide solution and BSA solution were slowly added, followed by the addition of a 10-fold molar excess of the SMCC solution to ensure that the final content of organic cosolvent was less than 5%. After reacting for 2 h at room temperature under stirring, excess reactants and byproducts were removed by gel filtration using a Sephadex G25 desalting column with phosphate buffer. The obtained coupling product is used as an immunogen to prepare an antibody against HbA1c with the appropriate sensitivity and specificity.

2.3. Preparation of acridinium ester-labeled anti-HbA1c antibodies

Acridinium ester was dissolved in DMSO to a concentration of 1 mg/mL. According to the molar ratio of HbA1c antibody:acridinium ester = 1:10, the HbA1c antibodies were diluted to a concentration of 1 mg/mL with 50 mM phosphate buffer at pH 7.6,

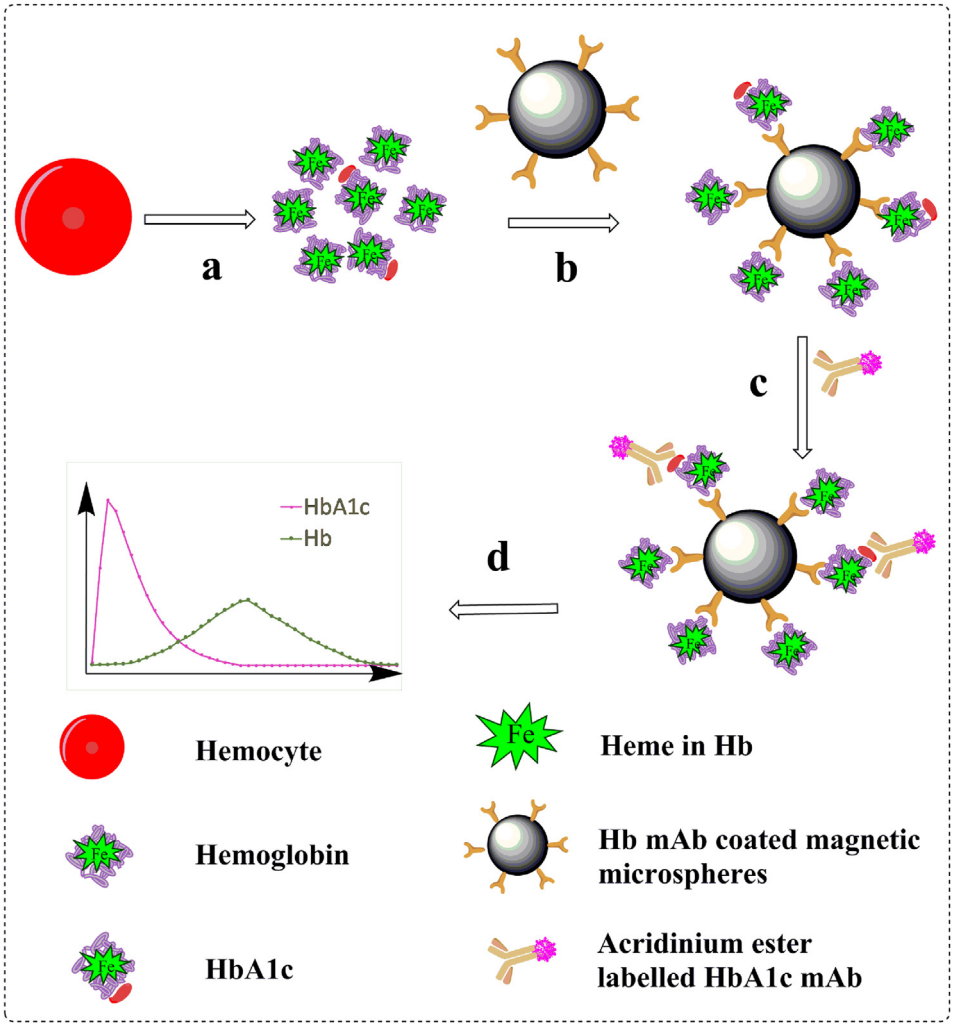

Fig. 1. Schematic illustration of the multiple chemiluminescence assay for HbA1c/Hb detection in whole blood samples. a: Erythrocytes were lysed to release Hb and HbA1c by denaturing agents containing surfactants. b: Magnetic beads coated with Hb antibodies were added to capture the total Hb. c: Acridinium ester-labeled anti-HbA1c antibodies were added to recognize HbA1c. d: Height and time of the chemiluminescence peak of the acridine eater and heme simultaneously triggered with trigger solution were utilized for multiple detections of HbA1c and Hb.

and acridinium ester solution was added. After reaction at 37 °C for 1 h, the unbound acridine was removed by dialysis.

2.4. Preparation of magnetic nanoparticles coated with anti-Hb antibodies

Magnetic nanoparticles can be used as a solid phase to capture targets [28–30] and detect various targets [31–35] and for application in portable nucleic acid detection [36,37] and issues of interest, such as cytotoxicity to cancer cells [38,39] and water treatment [40]. In this research, magnetic nanoparticles were applied to separate total Hb. According to the mass ratio of carboxyl magnetic nanoparticles (EDC: NHS = 1: 0.5: 0.5), EDC and NHS were added into carboxyl magnetic microspheres suspended in MES buffer to activate the carboxyl groups, and the mixture was washed three times after reacting at 37 °C for 30 min. The anti-Hb antibodies were slowly dropped into the dispersed magnetic microspheres according to the mass ratio of the anti-Hb antibodies: magnetic nanoparticles = 1: 20. After full stirring at 37 °C for 12 h, 0.2% w/v BSA and 0.2% w/v glycine solution were added to the reaction solution to block the remaining sites on the magnetic microspheres. After full stirring for another 1 h, the products were washed 3 times. Finally, magnetic nanoparticles were resuspended in phosphate buffer containing 2% w/v BSA at a working concentration of 2.5 mg/mL.

2.5. Preparation of a chemiluminescent biosensor for the detection of Hb

Considering that the heme in Hb has a strong chemiluminescent activity in the reaction involving hydrogen peroxide, the chemiluminescence of heme with a hydrogen peroxide-sodium hydroxide system was investigated for quantitative analysis of Hb. The interaction of H_2O_2 with Hb in NaOH solution was performed on a MAGICL 6000 system, as shown in Fig. 2. Ten microliters of pretreated Hb solution was pretriggered in a reaction cup for 3 s by trigger solution A, which contained 0.5% w/v hydrogen peroxide and 0.2 mol/L nitric acid, and trigger solution B, which contained 0.35 mol/L sodium hydroxide, and a certain concentration of surfactants was also added to the reaction mixture. Surfactants, such as 0.2% w/v Triton-100, Tween-20, and CTAC in trigger solution B, were added and used as the catalyst solution to optimize the chemiluminescent reaction conditions. Upon exposure to basic pH and peroxide, acridinium and heme underwent a decomposition

reaction with the release of photons of light as products (chemiluminescence). The emitted light was collected by a "light pipe" and directed to a photomultiplier tube (PMT), where the signals were amplified and processed. To yield the relative luminescent units (RLU) for the main control board, the signal was summed within 3 s by the photon counter and considered the final read result.

2.6. Samples preparation

The denaturing agents for sample preparation were added to enable hemolysis to expose the glycated residue of HbA1c so that it was accessible to anti-HbA1c antibodies. Fresh whole blood samples were placed and mixed on MAGICL6000 before pretreatment. The analyzer was automatically fed 2 μL of whole blood samples, 198 μL of denaturing agent containing 20 mmol/L CTAB and 1 mol/L ammonium chloride was added, and the blood cells were lysed to increase the detection sensitivity of HbA1c.

2.7. Immunoassay

The following operations were all performed by the MAGICL6000 chemiluminescence analyzer. First, 10 μL of the pretreated samples, 10 μL of magnetic beads coated with anti-Hb antibody (2.5 mg/mL), and 100 μL of acridine-labeled HbA1c antibody (0.4 μg/mL) were added to the reaction cup and then shaken, mixed, incubated at 37 °C for 10 min, and washed 3 times by magnetic separation. Finally, trigger solutions were added to the reaction mixture, and the resulting chemiluminescent reaction was measured based on RLU. A direct relationship was observed between the amount of HbA1c in the sample and the RLU detected by the MAGICL6000.

2.8. Analysis performance evaluation

2.8.1. Reproducibility

The reproducibility study of the HbA1c assay was conducted on the MAGICL6000 chemiluminescence analyzer using the proposed method. The study was performed following CLSI guideline EP5-A3, "Evaluation of Precision Performance of Quantitative Measurement Methods". Five whole blood samples obtained from the outpatient clinic at the Second Hospital of Nanjing were tested using the study design of 20 days × 2 runs per day × 2 replicates per sample (Sample 1: 3.5%, Sample 2: 4.5%, Sample 3: 6.5%, Sample 4: 8.5%, and Sample 5: 10.5%).

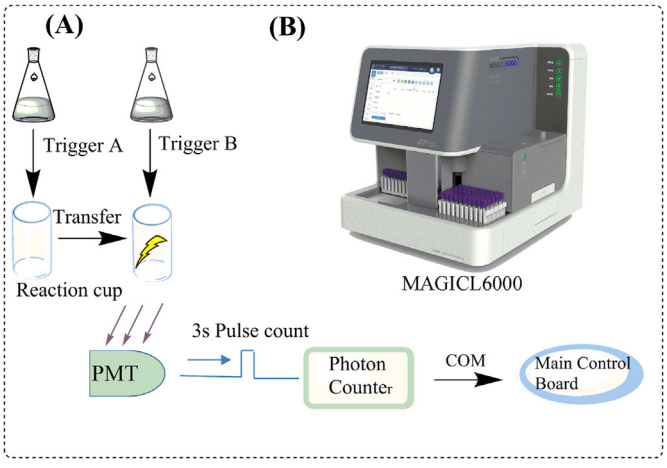

Fig. 2. (A) Schematic diagram of the multiple chemiluminescence detection system. (B) Image showing the appearance of MAGICL6000.

2.8.2. Comparison with the commercial HbA1c HPLC method

The multiple chemiluminescence method was compared with the commercial HbA1c method (BIO-RAD D10, HPLC) based on the CLSI-EP 9 guideline. Blood samples from both healthy and type 2 diabetes mellitus human patients with HbA1c levels ranging from 3.8% to 13.0% were obtained from the Second Hospital of Nanjing and tested over five days both on the proposed system and the BIO-RAD system. The correlation coefficient and regression equation methods were applied to evaluate the correlation between the two methods, and the 95% confidence interval and the mean relative error were estimated by the Bland–Altman method to determine the consistency.

2.9. Statistical analysis

A statistical analysis with a completely randomized design was conducted with SPSS 17.0 software based on the one-way analysis of variance (ANOVA) procedure. MedCalc Software (MedCalc, Mariakerke, Belgium) was applied to analyze the Bland–Altman plot and Passing–Bablok regression. The results of all data analyses were reported as the mean ± SD, and differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Quantification of Hb by chemiluminescence biosensor

The chemiluminescence response is linearly proportional to the amount of total Hb in the sample since Hb containing four iron heme groups acts as a substrate for the chemiluminescence reaction in the presence of hydrogen peroxide. As shown in Fig. 3A, the chemiluminescence produced by the oxidation of heme may be roughly attributed to the following two events [41–44].

1. Formation of a dioxetane intermediate through the reaction of unsaturated lipids with singlet oxygen. Decomposition of dioxetanes might yield excited carbonyl compounds that emit in the blue region upon relaxation to the ground state (1) [45].
2. Production of Fe(III)-heme–$O_2\cdot$ and superoxide radicals through the reaction of hydrogen peroxide with heme (2) and (3). Singlet oxygen can be produced by a series of reactions involving superoxide radicals that enhance the intensity of chemiluminescence. The α-methene bridge of Fe(III)-heme could be broken by the intermediate Fe(III)-heme–$O_2\cdot$ to produce a ferribile pigment and ferribiliverdin [44,45]. The destruction of the π-conjugated system of the porphyrin ring means that the unsaturated double bonds from the ferribile pigment and ferribiliverdin are more easily attacked by singlet oxygen, thus strengthening the first event mentioned above.

In addition, the surfactant may act as an enhancer of the chemiluminescent reactions of heme in Hb and H_2O_2. When heme reacts with H_2O_2 in an alkaline solution, chemiluminescence of certain intensity can be detected, and the reaction rate and intensity of the chemiluminescence will be affected by different surfactants. Measured with a MAGICL6000 chemiluminescence analyzer, the chemiluminescence curves of heme and acridine ester triggered with trigger solutions containing different kinds of surfactants are shown in Fig. 3B ~ D, and the luminosity sequence of heme and acridinium ester was as follows (the integral time was set to 3 s): CTAC > Triton X-100 > Tween-20. Both acridine ester and heme triggered by H_2O_2 were both affected by surfactants. When CTAC was used as a surfactant, the height and time of the emission peak of acridine ester and heme were different, suggesting that a time-resolved principle can be utilized for multiple detections of

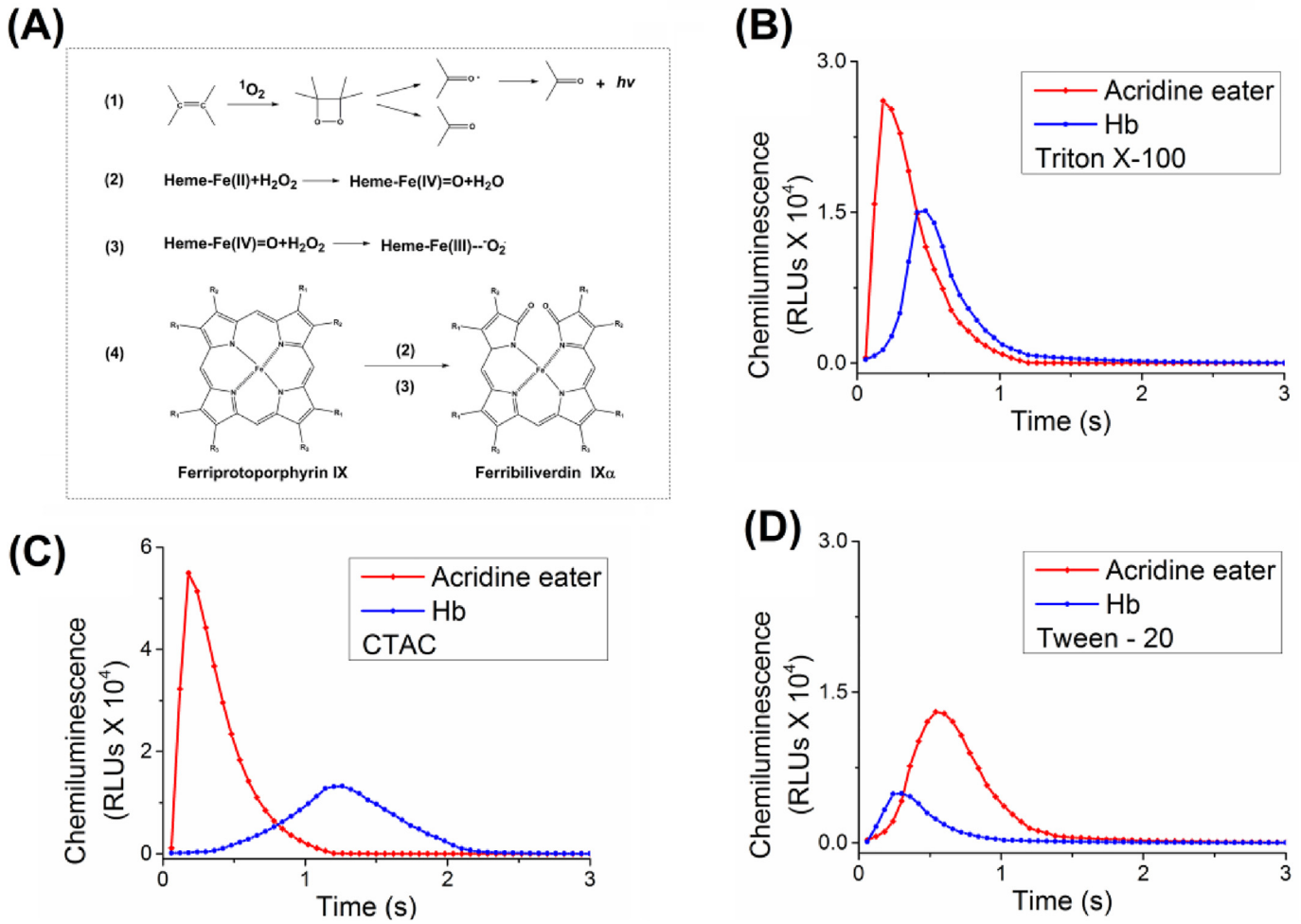

Fig. 3. (A) Schematic illustration of the mechanism of Hb chemiluminescence. (1) Formation of a dioxetane intermediate through the reaction of unsaturated lipids with $^1O_2$. Decomposition of dioxetanes might yield excited carbonyl compounds that emit in the blue region upon relaxation to the ground state. (2) and (3) Mechanism for generating superoxide during the reaction of H_2O_2 with heme could involve the formation of a ferrylheme intermediate, which can then react with a second molecule of H_2O_2 to produce Fe(III)-heme and superoxide. (4) α-Methene bridge of Fe(III)-heme was broken by the intermediate Fe(III)-heme-$^{\cdot}O_2^-$ to produce ferribile pigment and ferribiliverdin. R_1 = methyl; R_2 = vinyl; R_3 = acetoxyl. The chemiluminescence curves of heme and acridine ester were triggered with trigger solutions containing (B) Triton X-100, (C) CTAC, and (D) Tween-20. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

HbA1c and Hb. Although the two chemiluminescence signals will overlap between 0.5 s and 1 s, the number of photons within 0.5 s can still be used to indicate the chemiluminescence intensity of acridine ester, while the number of photons after 1 s can be used to indicate the concentration of ferriporphyrin, which is also the concentration of total Hb.

3.2. Multiple detections of HbA1c and total Hb based on time-resolved chemiluminescence

HbA1c can also be detected with the sandwich immunoassay method using the same Hb chemiluminescent detection system since the anti-Hb antibodies coated on magnetic particles can capture total Hb, and acridinium ester-labeled anti-HbA1c antibodies can recognize HbA1c. Under the optimal conditions, two representative standard curves (RLU values against Hb concentrations of 15–150 μg/mL and against HbA1c concentrations of 2.0–20 μg/mL) were successfully obtained, as shown in Fig. 4.

From the established calibration curves, the concentration ratio of HbA1c to total Hb was estimated using the following equation for human whole blood samples: HbA1c% = HbA1c/total Hb × 100%. Therefore, the percentage of HbA1c in the blood samples can be calculated by simultaneously detecting the HbA1c and total Hb with the same detection system, which cannot be performed with other conventional methods. When using immunoassays to detect HbA1c/Hb, many commercial kits can only be applied to measure the concentration of HbA1c assuming that the concentration of Hb is a constant value or that HbA1c and Hb react in a constant ratio during the immune reaction. However, in our experiment, we found that the total amount of Hb was affected by many factors, such as the hematocrit value, hemolysis process, sampling error, or other random errors of the detection system. Therefore, an accurate concentration cannot be obtained if the total Hb concentration is not taken into account during HbA1c detection. Subsequently, we compared the performance of the scheme measuring only HbA1c and the scheme measuring both HbA1c and Hb.

3.3. Reproducibility

Five whole blood samples with different HbA1c/Hb concentrations were tested in duplicate on 20 different days (2 runs per day) using the proposed method and a MAGICL6000 chemiluminescence analyzer. The reproducibility was calculated based on recommendations from the CLSI guideline EP5-A3, as shown in Table 1. The repeatability %CV for the proposed multiple HbA1c and Hb method ranged from 1.22 to 2.21%, with a median value of 1.73%,

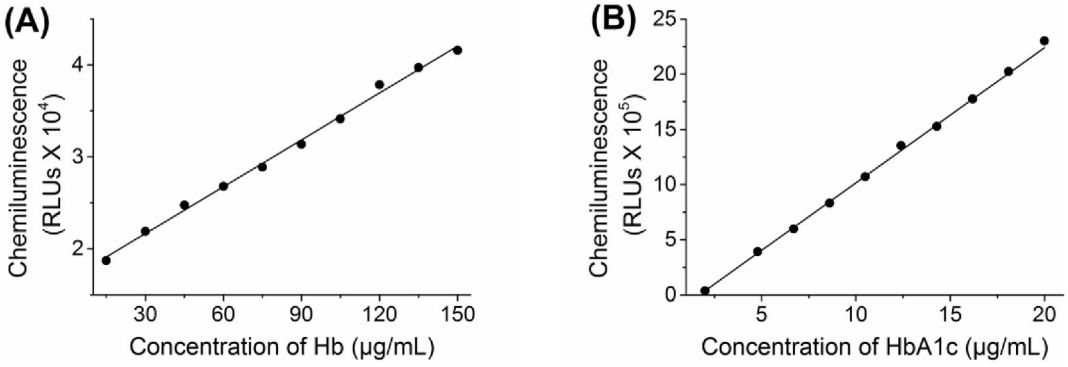

Fig. 4. Standard curves were obtained by the multiple chemiluminescence assay for (A) Hb and (B) HbA1c.

Table 1
Reproducibility of HbA1c detection of whole blood samples at 95% confidence limits.

Sample	Repeatability		Within-Laboratory Precision	
	%CV	95% CI	%CV	95% CI
1	2.21%	1.82%–2.83%	3.02%	2.57%–3.66%
2	1.94%	1.59%–2.48%	2.78%	2.38%–3.35%
3	1.67%	1.37%–2.14%	2.85%	2.45%–3.40%
4	1.63%	1.33%–2.08%	3.27%	2.80%–3.94%
5	1.22%	1.00%–1.56%	2.13%	1.85%–2.53%

while the within-site reproducibility %CV ranged from 2.13 to 3.27%, with a median value of 2.81%. Moreover, it should be noted that the present multiple detection method for HbA1c and total Hb based on time-resolved chemiluminescence was carried out in one reaction cup, which avoided systematic errors caused by a series of instruments, such as repeated sample addition, sample pretreatment (lysis of red blood cells), immune reaction and washing. All the above results strongly demonstrated that the multiple chemiluminescence assay was established with very high reproducibility at all concentrations of HbA1c/Hb.

3.4. Comparison study

A comparison study was performed to validate the practical applicability of our newly developed method by measuring the HbA1c/Hb value in 228 human blood samples from both healthy and diabetic adults and comparing the findings with the results obtained with the conventional HPLC method using the BIO-RAD D10 system. As shown in Fig. 5, the regression analysis comparing

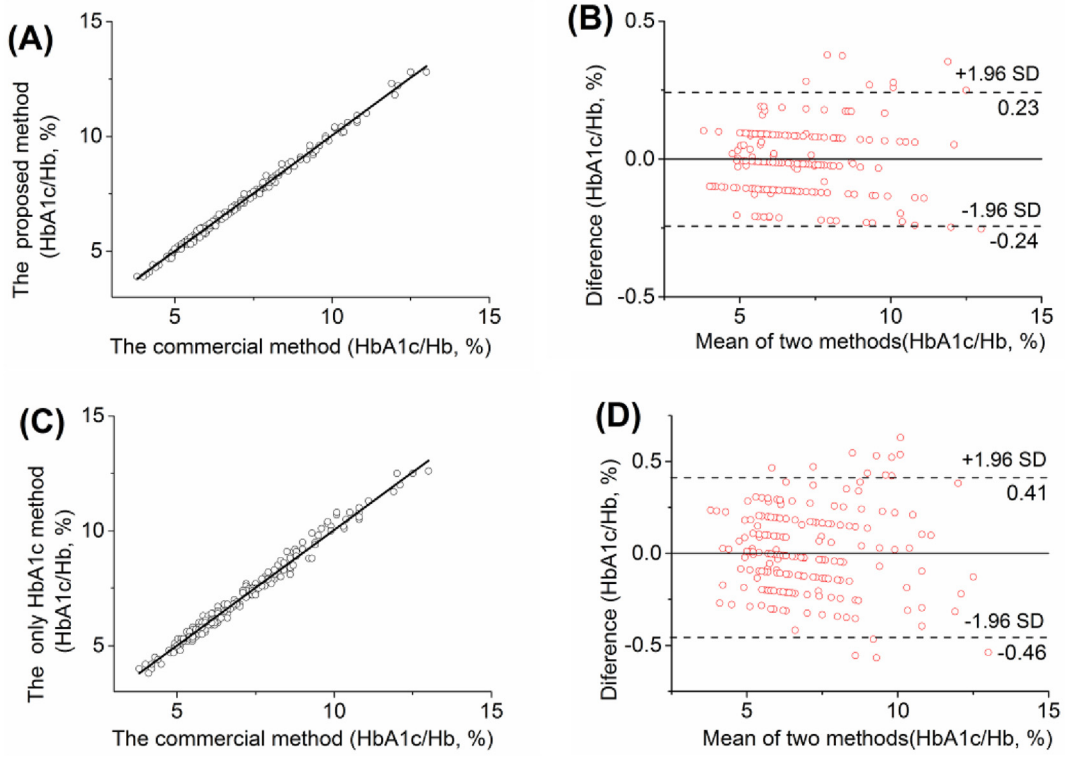

Fig. 5. Comparison study between the multiple chemiluminescence assay and clinical HPLC method (BIO-RAD D10) for HbA1c/Hb detection. (A) Correlation analysis; (B) Bland–Altman analysis. Comparison study between only the HbA1c method and the clinical HPLC method (BIO-RAD D10) for HbA1c/Hb detection. (C) Correlation analysis; (D) Bland–Altman analysis.

the results obtained by the proposed HbA1c/Hb multiple chemiluminescence with the predicated assay results yielded a slope of 1.00605 and a correlation coefficient of 0.9959, and the Bland–Altman analysis showed that the 95% confidence interval of the mean absolute bias was between −0.24% and 0.23%. However, with only the HbA1c method, the square of the correlation coefficient was 0.9851 and the mean absolute bias at the 95% confidence interval was between −0.46% and 0.41%, indicating that the HbA1c/Hb multiple chemiluminescence assay had a better consistency compared with the control assay and had better accuracy and great application prospects in clinical practice.

The hematocrit of different people varies, which leads to differences in total Hb. When the hematocrit value is very low and the amount of Hb is not enough to completely occupy the surface of the magnetic bead, HbA1c no longer reacts based on a real ratio, which ultimately leads to the inability to obtain an accurate proportion of HbA1c. Our time-resolved chemiluminescence immunoassay presented here not only allows for the simultaneous determination of both the HbA1c concentration and Hb concentration but also allows both signals to be measured in a single injection in a single reaction cup, which not only reduces the clinical testing time but also greatly reduces the errors associated with multiple additions and other issues caused by using different reaction cups.

4. Conclusions

In the present study, we developed a time-resolved multiple chemiluminescence assay approach for HbA1c/Hb detection with an immunoassay for HbA1c and a chemiluminescence biosensor for total Hb for the first time. Magnetic nanoparticles coated with anti-Hb antibodies can selectively capture the total Hb in human blood samples, and anti-HbA1c antibodies labeled with acridinium ester recognize HbA1c. After washing, the heme in Hb and acridinium ester indirectly labeled on HbA1c simultaneously reacted with H_2O_2 in alkaline solution to generate chemiluminescence. The height and time of the chemiluminescence peaks of acridine ester and iron porphyrin can be distinguished by optimizing the surfactant, thus allowing for the simultaneous determination of the concentration of total Hb and that of HbA1c. According to the performance analysis, the established method has excellent reproducibility and accuracy due to the advantages of multiple detections, which avoids the errors caused by the repeated addition of samples and the use of different reaction cups. The method established in this paper can not only be applied to detect HbA1c/Hb but also provides a new strategy for time-resolved chemiluminescence analysis. Because the reaction kinetic characteristics of acridine ester and heme can be distinguished, these two probes can be used to label any analytes that need to be detected simultaneously, which may have great prospects in the field of clinical diagnosis.

CRediT authorship contribution statement

Huan Zhao: Writing – original draft. **Xinghan Qiu:** Part Experiment. **Enben Su:** Methodology. **Li Huang:** Part Experiment. **Yunfeng Zai:** Formal analysis. **Yuan Liu:** Formal analysis. **Hui Chen:** Part Experiment. **Zunliang Wang:** Conceptualization. **Zhu Chen:** Conceptualization. **Song Li:** Writing – review & editing. **Lian Jin:** Funding acquisition. **Yan Deng:** Funding acquisition. **Nongyue He:** Supervision.

Declaration of competing interest

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

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