

The design of a homocysteine fluorescent probe based on Rhodamine B and its responsiveness in the serum of cerebral infarction patients

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Abstract. High levels of homocysteine (Hcy) is closely associated with the onset of cerebral infarction. The present study aimed to synthesize a novel Hcy probe based on Rhodamine B, named S1-4, a new compound that has not been previously reported. This probe exhibited good linear range under physiological fluid viscosity and pH; it has good selectivity for Hcy, and is able to avoid interference from other amino acids and metal ions. This probe can effectively measure the level of Hcy in the blood sera of healthy people and in patients with transient cerebral ischemia and cerebral infarction. However, satisfactory specificity and sensitivity to Hcy was not achieved according to receiver operating characteristic curve analysis. Overall, results from the present study suggested that following further optimization, this probe may be potentially applied in the diagnosis of cerebral infarction.

Introduction

Cerebral infarction, also known as acute ischemic stroke, is a common acute cerebrovascular disease that is harmful to the health of the elderly and the young (1). It is characterized by high rates of recurrence (26% within 5 years and 39% within 10 years), disability (19% of patients within 3 months and 38% within 12 months) and mortality (114.8/100,000) in China (2,3),

which seriously affects the quality of life of patients, as well as causing substantial emotional and economic burden to both the patients and their families. Developed countries worldwide, including China, have a rapidly aging society; therefore, effective detection techniques for the early screening, risk prediction, diagnosis and prognosis of cerebral infarction are urgently needed (3,4). Clinical and epidemiological data have confirmed that homocysteine (Hcy) is an important risk factor for cardiovascular diseases and is closely associated with the pathogenesis of cerebral infarction (5). The level of Hcy in the plasma of patients with cerebral infarction is significantly higher compared with that of the healthy population (6,7). Therefore, efficient methods of monitoring Hcy levels provides a potentially useful avenue for the early diagnosis, prevention and treatment, as well as prognosis, of patients affected by cerebral infarction.

Materials and methods

Synthesis of the probe. The Hcy probe was synthesized according to the pathway illustrated in Fig. 1. Briefly, 4-formylbenzoic acid ('I'; 15.0 g, 0.1 M) was dissolved in 100 ml of dichloromethane, and 5 drops of N,N-dimethylformamide was added as a catalyst. The reaction was cooled in an ice bath to approximately 0°C, before oxalyl chloride ('a'; 19.05 g, 0.15 M) was added dropwise. The reaction continued under rotary distillation at room temperature for 2 h to remove the solvent and excess oxalyl chloride. The 4-formylbenzoyl chloride ('II') obtained was used directly for the next step without purification. In a reaction flask, 50 ml of ethylene glycol ('b') was added and cooled to approximately 0°C in an ice bath. 4-Formylbenzoyl chloride was gradually added to the ethylene glycol, and the reaction was continued under rotary distillation at room temperature for 2 h to remove excess ethylene glycol. Crude 2-hydroxyethyl 4-formylbenzoate ('III') was then purified by silica gel column chromatography and eluted using petroleum ether/ethyl acetate (2:1) as the eluent, and a colorless, waxy solid (15.7 g, 80.93%) was obtained. Rhodamine B ('IV'; 2.39 g, 0.005 M) was dissolved in 30 ml of dichloromethane before the sequential

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addition of 2-hydroxyethyl 4-formylbenzoate (1.16 g, 0.006 M), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (0.96 g, 0.005 M) and 4-(dimethylamino)pyridine (0.12 g, 0.001 M). The reaction was carried out at room temperature for ~28 h, and was monitored using thin-layer chromatography with dichloromethane/methanol (10:1) as the solvent. This reaction system was subjected to rotary distillation to remove the solvent dichloromethane. The compound was dissolved in 2 ml dichloromethane and chromatography for purification was performed using a vitreous silica gel column (column diameter, 1.5 cm; column length, 70 cm; HuaSheng Chromatography, Jiangyin, China at a flow rate of 2 ml/min at room temperature. Dichloromethane/methanol (50 ml; 10:1) was used as the eluent. Finally, N-(6-(diethylamino)-9-(2-((2-(4-formylbenzoyloxy)ethoxy)carbonyl)phenyl)-3H-xanthen-3-ylidene)-N-ethyl-ethanaminium chloride ('V' also known as S1-4; 0.471 g, 14.4%) was obtained, named thereafter as S1-4. All reagents mentioned above were purchased from Sigma-Aldrich (Merck KGaA). The compound structure 'V' was dissolved by deuterium oxide and analyzed using ^1H -Nuclear magnetic resonance (^1H -NMR; 400-MR DD2; 400 MHz; Agilent Technologies, Inc.) and an electron ionization-high resolution mass spectrometry (EI-HRMS; Micromass[®] GCT Premier[™]; Waters Corporation).

Determination of Hcy probe fluorescence linearity. The Hcy probe (10 μM) was prepared in PBS (Beijing Solarbio Science & Technology Co., Ltd.). In a 96-well black microplate, 0, 1, 2, 5, 10, 20, 50 and 100 μM Hcy (Beijing Solarbio Science & Technology Co., Ltd.), 100 μl in human-based serum (cat. no. LUN5048; Randox Laboratories, Ltd.), was mixed with 100 μl of the working solution of the Hcy probe. Following incubation at 37°C for 30 min, fluorescence intensity was measured at the excitation wavelength of 280 nm and emission wavelength of 590 nm using a microplate reader (Thermo Fisher Scientific Inc.), from which a standard curve of concentration vs. fluorescence was generated.

Effect of fluid viscosity and pH on the response of the probe. The working solution of the probe (10 μM) was prepared in PBS. In a 96-well black microplate, 100 μl of 20 μM Hcy with different fluid viscosities (glycerol/water=1.005-219 cp) were added to 100 μl of the working solution of the probe. Following incubation at 37°C for 30 min, fluorescence intensity was measured at an excitation wavelength of 280 nm and emission wavelength of 590 nm to analyze the effect of fluid viscosity on probe responsiveness using a microplate reader (Thermo Fisher Scientific Inc.). To measure the effect of pH on the response of the probe, buffers at pH 6.5-8.5 were prepared using HCl, NaOH and PBS before 10 μM of the probe was added to 20 μM Hcy. Following incubation at 37°C for 30 min, probe response was measured by the same method using a microplate reader (Thermo Fisher Scientific, Inc.).

Determination of probe selectivity. Considering the complex intracellular environment, various interfering substances, such as the main amino acids and other small molecules, were added to the probe mixture, including various amino acids derivatives [cysteine (Cys); Hcy; N-acetylcysteine; glutathione (GSH); arginine; asparagine; aspartic acid; β -alanine; dithiothreitol;

glutamine; glutamic acid; glycine; histidine; isoleucine; L-alanine; leucine; lysine; methionine; phenylalanine; proline; serine; threonine; tryptophan; tyrosine; and valine; all 1 mM], metal ions [potassium (K^+); sodium (Na^+); calcium (Ca^{2+}); magnesium (Mg^{2+}); zinc (Zn^{2+}); aluminum (Al^{3+}); cadmium (Cd^{2+}); manganese (Mn^{2+})], and Na_2SO_3 and H_2S (made from Na_2S ; 1 mM for both). The working solution of the probe (10 μM) was prepared in PBS. In a 96-well black microplate, 100 μl of each of the substances mentioned above were added to the working solution of the probe. Following incubation at 37°C for 30 min, fluorescence intensity was measured at an excitation wavelength of 280 nm and emission wavelength of 590 nm to analyze probe selectivity in the presence of interfering substances. All reagents mentioned above were purchased from Beijing Solarbio Science & Technology Co., Ltd.

Detection of Hcy in the serum using the probe and a biochemistry analyzer. A total of 50 patients with cerebral infarction (CI group) and 50 patients with transient cerebral ischemia (TIA group) diagnosed in Tianjin Huanhu Hospital (Tianjin, China) between May 2016 and November 2017 were enrolled into the present study. A total of 50 healthy volunteers were classified into the normal (Norm) group. All participants in the experiments were matched for age (TIA, 60.1 \pm 11.2; CI, 62.7 \pm 9.1; Norm, 61.1 \pm 9.6) and sex (25 males and 25 females in each group). This study was approved by the medical ethics committee of Tianjin Huanhu Hospital, and followed the principles according to the Declaration of Helsinki; all patients provided informed consent before the experiments.

The inclusion criteria were as follows: i) Patients in the CI and TIA group were diagnosed within 24 h following the onset of symptoms according to the standard clinical criteria for CI and TIA, with supporting brain images produced either by computed tomography or magnetic resonance imaging and magnetic resonance angiography. The venous blood of Norm, CI and TIA groups were collected within 24 h of admission into hospital into an sterile and impermeable container before being frozen immediately at -80°C after collection; and ii) asymptomatic patients undergoing physical examinations who did not suffer from previous or current cardiovascular and cerebrovascular diseases were enrolled into the healthy control (Norm) group. The exclusion criteria were as follows: i) Patients with intracerebral hemorrhage or other brain injuries, atherosclerosis, heart failure, respiratory failure, renal failure, severe liver dysfunction, malignant tumors, autoimmune disease, diabetes mellitus or any other metabolic diseases, intestinal disorders, or other malignant disease; and ii) admission >24 h after stroke onset.

A total of 5 ml venous blood was collected from patients in each of the groups and centrifuged at 300 \times g for 10 min at room temperature. Hcy content in the serum was measured using a Biochemistry Analyzer (Beckman Coulter, Inc.). Dithiothreitol (1 M, 5 μl ; Sigma-Aldrich; Merck KGaA) was added to 95 μl of the serum and incubated at 37°C for 30 min, followed by the addition of the probe solution (10 μM) and another round of incubation at 37°C for 30 min. Fluorescence intensity was measured at the excitation wavelength of 280 nm and emission wavelength of 590 nm using a microplate reader (Thermo Fisher Scientific, Inc.). Using the fluorescence intensity data, a correlation and receiver operating characteristic

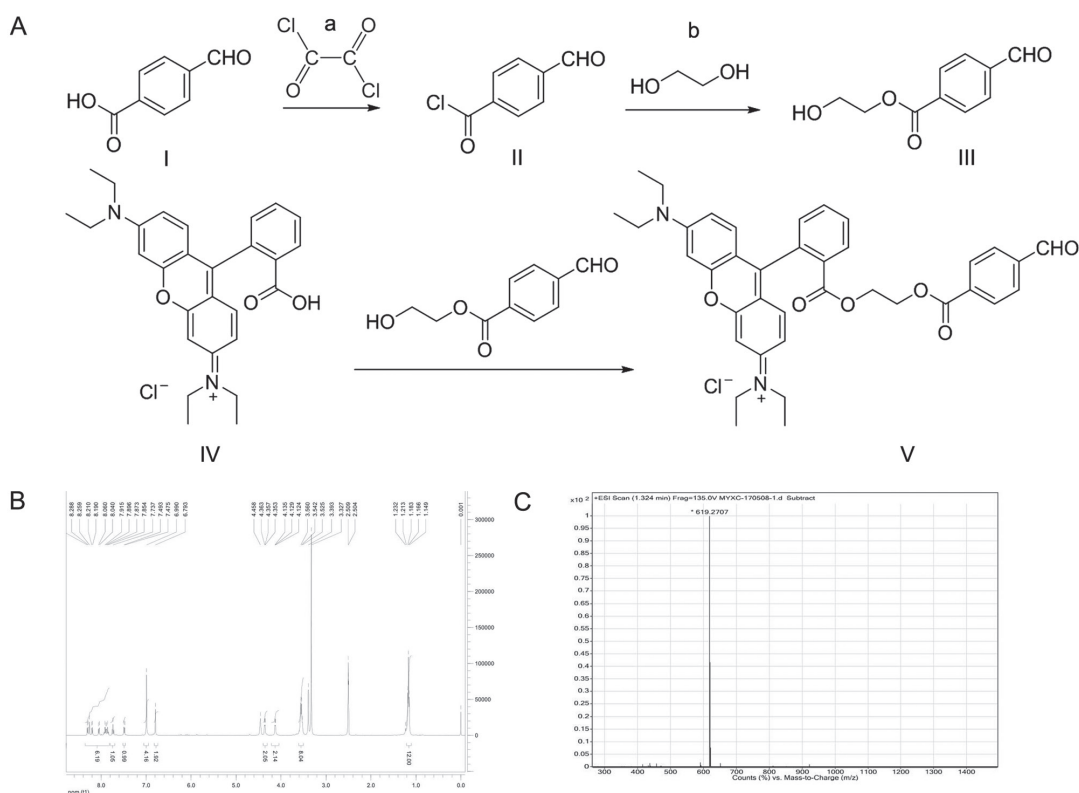


Figure 1. Synthesis pathway of probe. (A) The compound S1-4 (also known as V) was synthesized using the synthesis pathway. The structure of probe was identified by (B) ¹H-nuclear magnetic resonance spectroscopy and (C) electron ionization-high resolution mass spectrometry.

(ROC) curve was produced to compare the accuracy, specificity and sensitivity of probe in biological samples.

Statistical analysis. Data are represented as mean \pm SD and were analyzed using SPSS 11.0 software (SPSS, Inc.). One-way ANOVA and the Tukey post hoc test was used for general measurement data. Pearson's correlation coefficient was used for correlative analysis. ROC curve was calculated using GraphPad Prism 7.0 (GraphPad Software, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

The structural identification of the probe. To illustrate the structure of the probe, the following results were obtained using ¹H-NMR and EI-HRMS spectroscopy: ¹H NMR (400 MHz, DMSO-D₆) δ 10.07 (s, 1H, -CHO), 8.30-7.90 (m, 6H), 7.74 (t, $J=7.6$ Hz, 1H), 7.49 (d, $J=7.2$ Hz, 1H), 6.99 (m, 4H), 6.79 (s, 2H), 4.35 (t, $J=2.4$ Hz, 2H), 4.13 (t, $J=2.4$ Hz, 2H), 3.55 (q, $J=7.2$ Hz, 8H), 1.17 (t, $J=6.8$ Hz, 12H; Fig. 1B); EI-HRMS ($C_{38}H_{39}N_2O_6^+$; Fig. 1C). Molecular weight: 619.2707. Thus, S1-4 was identified as the target compound.

Linearity of Hcy probe detection. Following probe synthesis, to explore the correlation between fluorescence intensity and Hcy concentration, the fluorescence intensity of the S1-4 probe in the presence of ascending concentrations of Hcy was measured. A plot displaying fluorescence intensity of the probe vs. Hcy concentration was obtained (Fig. 2). At the concentration range of 0-100 μ M, fluorescence intensity of the probe

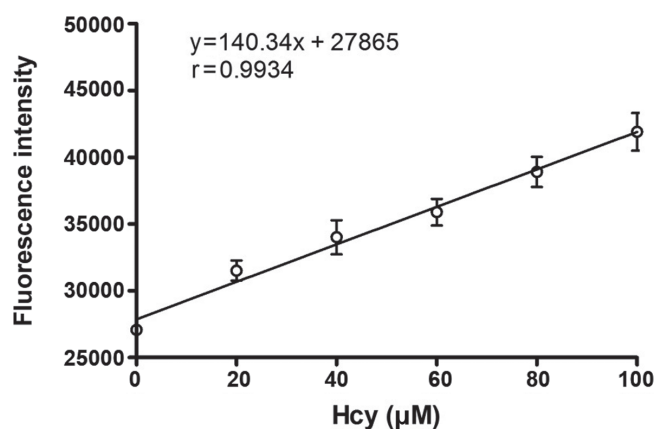


Figure 2. Linearity of the detection of Hcy by the probe. The graph demonstrates that there was a positive correlation between fluorescence intensity of the probe and Hcy concentration in the 0-100 μ M range. Hcy, homocysteine.

exhibited a good linear relationship with Hcy concentration ($r=0.9934$).

Effect of fluid viscosity and pH on the responsiveness of the probe. Hcy at a concentration of 20 μ M was selected as it is the concentration found in the majority of healthy individuals (8). To characterize the probe under physiological conditions in the human bloodstream (including blood, pH and viscosity), following reaction with 20 μ M Hcy, the fluorescence intensity of the S1-4 probe did not appear to be affected by the increasing viscosities of the reaction mixture (glycerol/water, 1.005-219 cp;

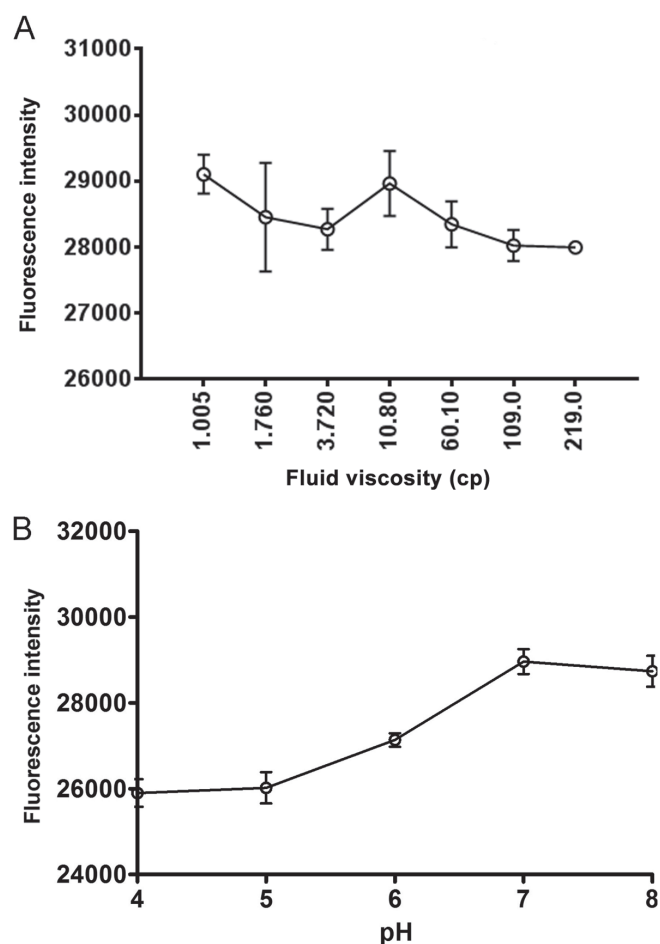


Figure 3. Effects of fluid viscosity and pH on the responsiveness of the Hcy probe. (A) The graph demonstrates that fluorescence intensity of the probe in response to Hcy was not affected by the different viscosities (glycerol/water, 1.005–219 cp). (B) The graph demonstrates that the fluorescence intensity exhibited stable responsiveness to Hcy (20 μ M) at pH 7. Hcy, homocysteine.

Fig. 3A). The viscosity range was selected as it spans the normal and abnormally high viscosities observed in healthy and patients with diseases, respectively (9). This suggested that probe activity is unlikely to be affected by changes in blood viscosity induced by changes to physiological functions or diseases (Fig. 4A). The probe exhibited the most fluorescence intensity (responsiveness) to Hcy at pH 7, which suggested that the activity of the probe is unlikely to be affected by changes in blood pH induced by changes to physiological functions or diseases (Fig. 3B).

Selectivity of the probe in the presence of different biological analytes. None of the interfering substances tested, including amino acids and high concentrations of various metal ions (see Materials and methods), appeared to have adversely affected the fluorescence intensity of the S1-4 probe in response to 20 μ M Hcy (Fig. 4). These observations suggested that the responsiveness of the probe has good selectivity for Hcy as the fluorescence intensity of Hcy was significant greater compared with cysteine and glutathione.

Measurement of Hcy in human serum. The probe was used to determine if it was able measure serum Hcy levels in healthy people, in addition to patients in the TCI and CI groups. The

levels of Hcy between the three groups could be distinguished by the probe. The serum levels of Hcy, as measured by the probe, was significantly higher in the CI group compared with the Norm group (Fig. 5A) and correlated positively with corresponding biochemistry data as measured using a Biochemistry Analyzer (Fig. 5B). However, according to the ROC analysis, the specificity and sensitivity of the S1-4 probe was the same as the analysis by the Biochemistry Analyzer, which suggested that the S1-4 probe needs to be optimized in further studies in the CI group (Fig. 5C and D).

Discussion

Hcy is a sulfur-containing amino acid that cannot be synthesized *in vivo* and can only be produced by the catabolism of methionine (10). Under normal circumstances, the Hcy content in the blood is very low, and high concentrations of Hcy have been found to be an independent risk factor for coronary artery disease, cerebrovascular disease and peripheral vascular disease; the risk of developing these conditions are increased with the increase in Hcy concentration (11–14). Some developed and developing countries, including China, the United States and Japan have large and rapidly aging populations. Hcy is an important diagnostic marker for cerebral infarction (15); therefore, it is of great importance to develop an effective Hcy detection method with potentially beneficial values in clinical applications.

Existing Hcy detection methods, such as enzymatic methods, high performance liquid chromatography, capillary electrophoresis separation and immunoturbidimetry, all entail high detection costs and produce ambiguous data, because Hcy, GSH and Cys compounds all contain a sulfhydryl group. Therefore, new Hcy detection technologies that are accurate and efficient are an urgent need in the clinical setting.

In the present study, Rhodamine B was used as the parent compound, and a new compound that has not been previously reported was synthesized. It was found that the probe could selectively react with the amino group and the thiol group in Hcy. It was hypothesized that the fluorescent probe could measure the levels of Hcy and may be applied as a potential clinical detection method for cerebral infarction. The authors then found, that the spatial structure of S1-4 caused a change in the fluorescence intensity of the system, effectively reflecting the level of Hcy *in vitro*. Therefore S1-4 was researched as a priority in the present study. From the fluorescence intensity measurements, it was effectively demonstrated that the probe can enhance the fluorescence intensity of the reaction media with Hcy. This meant that Hcy could be selectively detected without off-target responses to cysteine and other amino acids, resulting in good selectivity.

Significant structural homology of the small molecule sulfhydryl-containing biological compounds meant that few studies have been able to report the direct detection of various sulfhydryl compounds (such as Cys and GSH) using small molecule fluorescent probes (16). The probe reported in the present study exhibited good water solubility and linear relationship with Hcy concentration, and also demonstrated good selectivity even in the presence of common amino acid and ions found in serum. Importantly, it could discriminate Hcy from other sulfhydryl compounds such as Cys and GSH, potentially avoiding off-target interference.

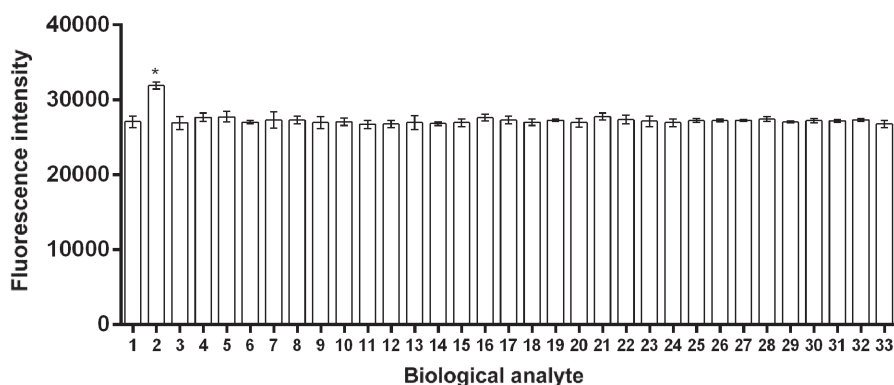


Figure 4. Selectivity of the probe in the presence of different biological analytes. The graph illustrates that there was a significantly increased fluorescence intensity of the probe in response to Hcy compared with other biological analytes. * $P < 0.05$ vs. the other biological analytes. 1, cysteine; 2, Hcy; 3, N-acetylcysteine; 4, glutathione; 5, arginine; 6, asparagine; 7, aspartic acid; 8, β -alanine; 9, dithiothreitol; 10, glutamine; 11, glutamic acid; 12, glycine; 13, histidine; 14, isoleucine; 15, L-alanine; 16, leucine; 17, lysine; 18, methionine; 19, phenylalanine; 20, proline; 21, serine; 22, threonine; 23, tryptophan; 24, tyrosine; 25, valine; 26, potassium (K^+); 27, sodium (Na^+); 28, calcium (Ca^{2+}); 29, magnesium (Mg^{2+}); 30, zinc (Zn^{2+}); 31, aluminum (Al^{3+}); 32, cadmium (Cd^{2+}); 33, manganese (Mn^{2+}). Hcy, homocysteine.

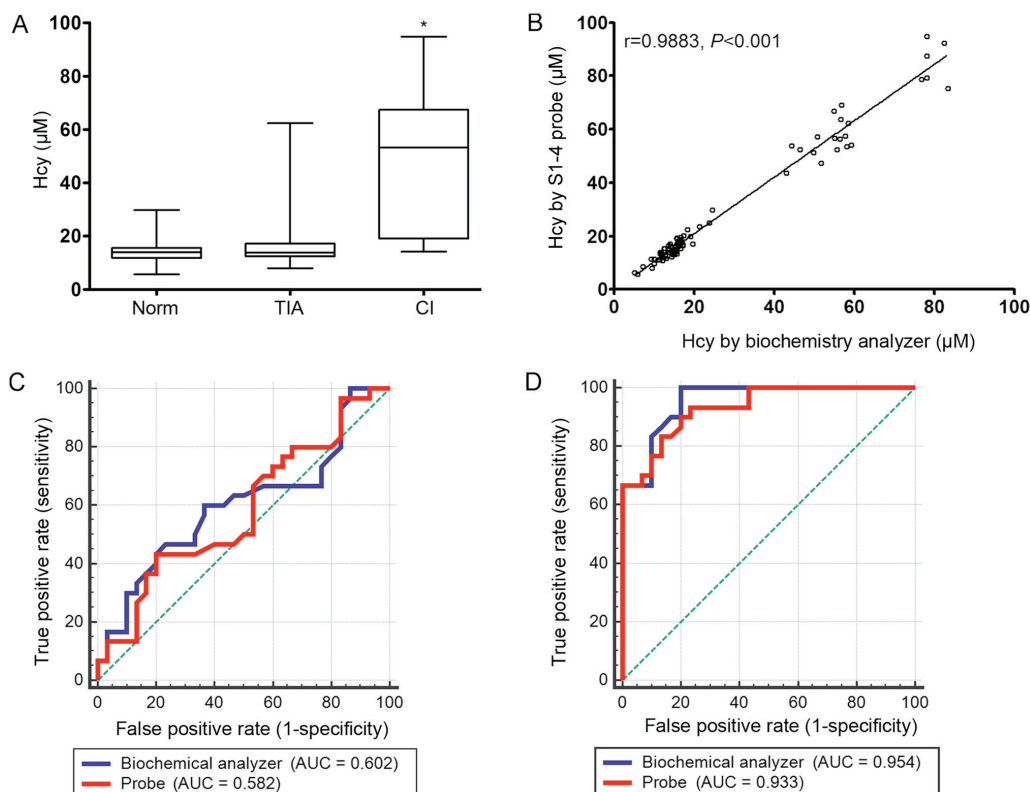


Figure 5. Measurement of Hcy in human serum. (A) The graph demonstrates that there was a significantly increased level of Hcy in patients in the CI group compared with patients in the Norm and TIA groups. * $P < 0.05$ vs. CI group. (B) The graph illustrates that there was a significant positive correlation between the levels of Hcy as measured using biochemistry and the probe method in the three groups. (C) The ROC analysis displays the specificity and sensitivity of the probe in the TIA group compared with the Biochemistry Analyzer. (D) The ROC analysis displays the specificity and sensitivity of the probe in the CI group compared with the biochemistry analyzer. CI, cerebral infarction; Hcy, homocysteine; Norm, normal; ROC, receiver operating characteristic; TIA, transient cerebral ischemia.

Clinically, this probe also effectively reflected the level of Hcy in serum of healthy people, as well as patients with TCI and CI. The measurements from clinical specimens demonstrated that the fluorescence signal of the probes in patients with CI was significantly higher compared with those in the healthy control group and in the TIA group; which were comparable

with those obtained from the Biochemistry Analyzer. This result supported the notion that this probe may have potential clinical application value.

In a recent study, Yang *et al* (17) reported a near-infrared fluorescent probe for Cys and Hcy detection using the colorimetry method. However, the probe proposed by that study

is suitable for monitoring and visualizing Cys and Hcy in cells, whereas the probe synthesized by the present study is only suitable for monitoring Hcy in blood sera. In addition, ROC curve analysis reported unsatisfactory probe specificity and sensitivity compared with data from the Biochemistry Analyzer, which indicated a need to optimize this probe for further study, such as by improving the chemical structure and linking to better vehicles. In the future, the number of specimens will need to be increased further, with the detection results compared with those obtained using existing established detection methods to develop a new type of Hcy detection probe. As diabetes mellitus, fatty liver disease and other metabolic diseases are also risk factors for cerebral infarction, the efficacy of the S1-4 probe in measurements of Hcy levels in patients with these diseases will also be explored further.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

YZ and YM conceived and designed the study. XZ acquired the data. XL performed the data analysis.

Ethics approval and consent to participate

This study was approved by the medical ethics committee of Tianjin Huanhu Hospital (Tianjin, China), in accordance with the Declaration of Helsinki; all patients provided informed consent prior to enrollment in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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