A novel enhanced chemiluminescent immunoassay (CLIA) for ultrasensitive and excellent precise determination of cardiac troponin I (cTnI) was reported. The method made full use of poly[(N-isopropyl acrylamide)-co-(methacrylic acid)] (P(NIPAM-co-MAA)) microspheres as new potential signal enhancers and magnetic fluorescent nanoparticles as internal standards for better precision. This protocol involved a sandwich format, in which the antigen in the sample was captured by the immobilized antibodies on the surface of magnetic fluorescent beads and recognized by the other antibodies labeled with acridinium ester (AE)-loaded P(NIPAM-co-MAA) microspheres. The combination of the remarkable sensitivity of the enhanced CLIA method and the use of P(NIPAM-co-MAA) microspheres as anti-cTnI carriers for acridinium ester signal amplification provided an extremely sensitive limit of blank (LoB) at 0.097 pg mL$^{-1}$, a limit of detection (LoD) at 0.116 pg mL$^{-1}$, and a limit of quantitation (LoQ) at 0.606 pg mL$^{-1}$, much greater than those achieved by the classical chemiluminescence immunoassay (CLIA, Getein). Moreover, the intra-day variable coefficient can be improved to 1.21–2.12%, and inter-day variability was 2.01–3.49% under the application of magnetic fluorescent beads as an internal standard. The sensitivity and precision have reached a high level, comparable with the current commercial detection kits. The results showed a good correlation with a commercial chemiluminescence assay (CLIA, Abbott), with a correlation coefficient of 0.9883. This proposed method has been successfully applied to the clinical determination of cTnI in the human serum.

**Introduction**

Acute myocardial infarction (AMI) remains a leading cause of morbidity and mortality worldwide, despite substantial improvements in prognosis over the past decade. The AMI occurs when a part of the heart muscle is damaged due to a lack of blood supply, possibly caused by blockage of a coronary artery. The myocardial injury is irreversible and 85% of heart damage progresses within the first two hours after the onset of a heart attack, and delayed medical treatment increases the probability of mortality. Therefore, early and accurate diagnosis of AMI and prompt treatment are critical to increasing the survival rate. Cardiac troponin (cTn) is an essential component in the diagnosis of myocardial injury. Troponin I is a subunit of the troponin complex, which plays an important role in the regulation of skeletal and cardiac muscle contraction. The complex consists of three subunits: troponin T (TnT), troponin I (TnI), and troponin C (TnC). These subunits are held together by non-covalent interactions. In the late 1980s, the contemporary cTnI (and cTnT) assays were able to detect cTn from the blood of patients at ng mL$^{-1}$ (μg L$^{-1}$) levels. In practice, these assays could reliably detect troponin only 3–6 hours after the onset of ischemic symptoms such as chest pain, meaning that cTns were considered to be late markers of AMI. In contrast, recent cTn (hs-cTn) assays with high sensitivity, the detection limit of which is pg mL$^{-1}$ (ng L$^{-1}$) rather than ng mL$^{-1}$, have made it possible to identify any myocardial injury, including those of AMI patients, within 1 to
3 hours. The 3-hour time saved is more conducive to rapid patient management. Cardiac troponin I (cTnI) is currently considered as the gold standard biomarker test for myocardial infarction.\(^8,9\) Moreover, cTnI measurements employing a new generation of high-sensitivity cTnI assays could be helpful for long-term risk stratification of different patient groups including patients with heart failure or acute coronary syndrome. Chemiluminescence immunoassay (CLIA) has been used as an attractive analytical method instead of enzyme-linked immunosorbent assay (ELISA) and point of care testing (POCT),\(^10\)\(^-\)\(^12\) because of its high sensitivity, rapidity of reaction, simple instrumentation, and wide dynamic range. However, the sensitivity of CLIA needs to be further improved to meet the demand in clinical disease diagnosis and treatment.\(^13\) An alternative approach for signal amplification in CLIA is to use an efficient enhancer or increase the number of labels conjugated to each antibody. Recently, many improvements including enhanced sensitivity and reduced detection time have been made in cTnI measurements.\(^14\)\(^-\)\(^16\)

The most recent international guidelines recommend that high-sensitive methods (HS-methods) should be preferred for the measurement of cardiac troponin I (cTnI) in patients admitted to the emergency department with acute coronary syndromes (ACS). The 2018 Expert Opinion from the American Association for Clinical Chemistry (AACC) and the International Federation of Clinical Chemistry (IFCC)\(^9\) recommends that HS-methods should satisfy two fundamental criteria. First, HS-methods should measure the 99th percentile upper reference limit (URL) of a healthy population with an imprecision (expressed as CV %) \(\leq 10\%\). Second, the assays should be able to detect cTnI concentration at or above the limit of detection (LoD) in at least 50% of healthy (normal) subjects. Ultrasensitive performances with excellent precision are very important for HS-cTnI laboratory methods, especially when the analyte is present at very low levels during the early stages of disease development. This article has introduced a novel ultrasensitive and enhanced CLIA based on the combination role with AE-labeling and P(NIPAM-co-MAA) as a new enhancer for further signal amplification. Also, the combination of magnetic and fluorescent properties in a single core/shell particle is a powerful new tool allowing manipulation by the magnetic field and calibrated by fluorescence to achieve better precision.

Hydrogels sensitive to pH and temperature have been extensively studied because of their engineering applications as well as their biomedical applications.\(^17\)\(^-\)\(^19\) The poly\([\text{N-isopropylacrylamide}-\text{co-(methacrylic acid)}]\) (P(NIPAM-co-MAA)) hydrogel is typical of gels that exhibit volume phase transitions with changes in temperature. In this work, the P(NIPAM-co-MAA) was obtained by copolymerizing NIPAM and MAA, and the acridinium ester (AE) was added to the polymer at the same time. After polymerization, the AE was wrapped in the microsphere with both carboxyl group and amphiphilic isopropylamide group on the surface. When the temperature is low, the isopropylamide group has strong hydrogen bond interactions with each other, which can lock the AE in the gel state, and the carboxyl group on the surface of the particle can be used to conjugate detection antibodies. The prepared AE-loaded P(NIPAM-co-MAA) provided stable chemiluminescent intensity, high chemiluminescent labeling efficiency, and low background.

Magnetic nanoparticles are used in a variety of biotechnology applications,\(^20\)\(^-\)\(^25\) most notably for cell sorting and assay separations.\(^26\)\(^-\)\(^30\) Several groups have reported successful synthesis and characterization of particles that possess both fluorescent and magnetic properties.\(^31\)\(^-\)\(^33\) In this study, we have demonstrated the methods to load magnetic nanoparticles with fluorophores and investigated their application as an internal standard in a sandwich immunoassay as shown in Fig. 1. Streptavdin immobilized on magnetic fluorescent nanoparticles, analyte, a biotinylated analyte-specific antibody and another analyte-specific antibody labeled with AE-P(NIPAM-co-MAA) microspheres complex reacted to form a sandwich immunocomplex. After washing, the heated trigger solutions were then added to the reaction mixture. Affected by heating, the hydrogen bonds in the AE-P(NIPAM-co-MAA) microspheres weaken, the gel collapsed and the AE exposed to the outside. Subsequently, the AE on the surface of the complex can be triggered and produced a stronger luminescence intensity. The resulting chemiluminescent reaction and fluorescence intensity in the magnetic nanoparticles were measured as a relative light unit (RLU) and Absorbance Unit (AU) respectively.

In this study, hydrogel microspheres sensitive to temperature as new potential signal enhancers and magnetic fluorescent nanoparticles as internal standards were used to establish a new CLIA method for the accurate diagnosis of cTnI in the human body. The low ratio of signal labels including enzymes, illuminant dyes to antibody results in a limited

![Fig. 1 Schematic illustration of the established sandwich-type cTnI-CLIA method.](image-url)
detectable background signal and better sensitivity of the traditional CLIA. This signal amplification technology was more challenging than that of previous work, because it made full use of the inner space of microspheres, which avoids the competition between antibody and acridine ester on the surface of microspheres, and the application of hydrogel allowed the internal signals being released. Furthermore, the fluorescence signal on the MFNPs is used as the internal standard to indicate the inaccuracy of the measurement system caused by random errors of magnetic beads during sampling or cleaning. Our results showed that the method had great advantages in sensitivity and precision compared with current commercial products, indicating that this assay could be used for the early and accurate detection of cTnI.

Experimental

Materials

Acridinium ester was purchased from Materwin (Shanghai, China). NHS-Biotin and Streptavidin were purchased from ThermoFisher Scientific (USA). All the antibodies were created by HyTest Ltd (Turku, Finland). A 2 × 2 pair of monoclonal antibodies (mAb) recognizing epitopes in the central region and at both ends of the cTnI molecule were used. The detection antibodies recognize the amino acid sequences 41–49 (19C7-mAb) and 18–35 (810-mAb). A mAb recognizing the epitope at 190–196 (MF4-mAb), and a mAb recognizing the epitope at 82–93 (560-mAb) were used as capture antibodies. Fluorescein isothiocyanate (FITC), rhodamine 110 and dichloromethane were all of analytical grade and purchased from Aladdin Reagent Co. (Shanghai, China). NHS-Biotin and Streptavidin were purchased from ThermoFisher Scientific (USA). All the antibodies were created by HyTest Ltd (Turku, Finland). A 2 × 2 pair of monoclonal antibodies (mAb) recognizing epitopes in the central region and at both ends of the cTnI molecule were used. The detection antibodies recognize the amino acid sequences 41–49 (19C7-mAb) and 18–35 (810-mAb). A mAb recognizing the epitope at 190–196 (MF4-mAb), and a mAb recognizing the epitope at 82–93 (560-mAb) were used as capture antibodies. Fluorescein isothiocyanate (FITC), rhodamine 110 and dichloromethane were all of analytical grade and purchased from Aladdin Reagent Co. (Shanghai, China). The commercial cTnI CLIA kit was provided by Getein Biotechnology Co. Ltd (Nanjing, China). Ferric chloride (FeCl3·6H2O), ferrous chloride (FeCl2·4H2O), ammonium hydroxide (25 wt%), styrene, 2,2′-azodiisobutyronitrile, butyl methacrylate, hexadecanol, absolute ethyl alcohol, disodium phosphate anhydrous, sodium dihydrogen phosphate anhydrous, and sodium chloride were purchased from Shanghai Chemical Reagents Company. Hexane (98%), oleic acid, N-isopropyl acrylamide (NIPAM, 99%), and acrylactic acid were purchased from Acrros Organics (USA). N,N′-Methylenebisacrylamide (MBA, 98%), methacrylic acid (MAA, 98%), potassium persulfate (K2S2O8, 99.5%), sodium dodecyl sulfate (SDS), N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC), bovine serum albumin (BSA), 2-[N-morpholine]-ethane sulphonic acid (MES, ultra-pure grade, 99.0%), and Tween-20 were purchased from Sigma–Aldrich (USA). Chemicals were of analytical reagent grade and deionized, and doubly distilled water was used throughout.

Preparation of acridinium-ester-loaded P(NIPAM-co-MAA) microspheres (AEPMs)

P(NIPAM-co-MAA) microspheres were synthesized according to the procedure reported by Alvarez-Lorenzo.34 Typically, 50 mg of SDS and 100 mg of K2S2O8 were dissolved in deionized water (100 mL) in a flask. 2 mL of NIPAM, 0.1 mL of MAA, and 0.05 mL of MBA were mixed in a tube before adding to the flask at room temperature and the solution obtained was purged with N2 gas under stirring for 30 min. The polymerization process was carried out for 8 h at 70 °C under a nitrogen atmosphere. Then, the mixed solution was purified by three repetitions of centrifugation at 10 000 rpm for 30 min and redispersed in 50 mL of deionized water. At room temperature, a dispersed solution of P(NIPAM-co-MAA) microspheres (100 mg) was mixed with SDS (10 mg) and AE (1 mg) for 1 h under stirring. The AE-loaded microspheres were centrifuged and dispersed in 10 mL of deionized water.

Preparation of AEP-membrane anti-cTnI antibodies

The AEP mixture was washed with a 0.05 M MES buffer (pH 5.5) and then centrifuged at 14 000g for 10 min. The clear supernatant was carefully removed, and the AEPs were resuspended in 1 mL of 0.05 M MES buffer (pH 5.5). 100 μL of 2 mg mL−1 NHS solution and 100 μL of 10 mg mL−1 EDC solution were added to AEPs and incubated at 37 °C for 0.5 h to activate the carboxylic acid groups on the microbeads. The AEPs were then washed with 0.05 M PBS buffer (pH 8.0) three times and 5 μg of anti-cTnI antibody (19C7/810) was added, followed by incubation at 37 °C for 2 h. 0.25% (w/v) bovine serum albumin (BSA) was added to the above test tube. The resultant mixture was incubated at 37 °C for 20 min to block the remaining active surface of AEPs.

Preparation of carboxyl-functionalized magnetic fluorescent nanoparticles (MFNPs)

The MFNPs were prepared by the following published procedure with a slight modification.35–38 75 g of FeCl3·6H2O and 30 g of FeCl2·4H2O were dissolved in 100 mL of deionized water under nitrogen gas with vigorous stirring at 75 °C. Then, 160 mL of ammonia was added rapidly into the solution, upon which the colour of the solution turned black immediately. After 2 hours, 8 mL of oleic acid was added and the suspension was kept reacting at 75 °C for 1 h. The magnetite nanoparticles were washed with deionized water until neutral. The magnetic nanoparticles modified by oleic acid were transferred in situ into 100 mL of hexane and a stable ferrofluid in hexane was obtained without obvious aggregates. The ferrofluid was mixed with 110 mL of styrene, 1.25 g of 2,2′-azobisisobutyronitrile, and 25 mL of butyl methacrylate, and the mixture was put into the ice bath with ultrasonication for 20 min to obtain the black dispersed solution. 1.5 g of SDS and 4.5 g of hexadecanol were dissolved in 100 mL of deionized water in a three-neck reaction flask equipped with a condenser under a nitrogen atmosphere and then heated to 55 °C for 20 min under stirring. The black solution prepared before was added into the three-neck reaction flask for pre-emulsification. After stirring for 30 min, 10 mg of FITC (10 mg mL−1, dissolved in dichloromethane), 2.5 g of KPS, and 1 mL of acrylactic acid were added, and then heated to 65 °C and kept at that temperature for 20 h. The magnetic fluorescent nanoparticles were col-
lected by centrifugation and cleaned by repeated centrifugation and ultrasonic dispersion in water.

Preparation of SA immobilized MFNPs

The SA immobilized MFNPs were prepared as follows. Briefly, 1 mL of a magnetic microbead slurry was placed in a test tube and washed with 0.05 M MES buffer (pH 5.5) three times. 100 μL of 2 mg mL\(^{-1}\) NHS solution and 100 μL of 10 mg mL\(^{-1}\) EDC solution were added to the tube and the resultant mixture was incubated at 37 °C for 0.5 h to activate the carboxylic acid groups on the microbeads. The beads were then washed three times and 200 μL of anti-cTnI antibody solution was added, followed by incubation at 37 °C for 3 h. Then, particles coated with SA were washed with TBST and resuspended with 1 mL of storage buffer (0.05 M TRIS containing BSA and ProClin 300) and the final concentration of particles was made to be 2.5 mg mL\(^{-1}\) for use.

Preparation of biotin-labelled antibodies

Anti-cTnI antibody (MF4/560) solution (155 μg) was placed in 0.5 mL centrifuge tubes and mixed well with label buffer (0.1 M phosphate-buffered saline (PBS) containing 0.15 M NaCl, pH 7.6). Then, a certain volume of 10 mg mL\(^{-1}\) NHS-biotin solution was added with the final volume being 150 μL. The antibody was labelled in a constant-temperature incubator (37 °C) for 0.5 h. Next, 20 μL of 5% w/v-lysine hydrochloride solution was added into the tube and incubated in the constant-temperature incubator (37 °C) for 30 min. Then, the reaction was terminated. The labelled mixture was loaded to the pre-balanced Sephadex G-25 desalting column for elution and collection of target antibodies. PBS (0.1 M) containing 0.15 M NaCl (pH 6.3) was used as elution buffer. The eluted components were collected with 500 μL in each tube and the samples were obtained. Finally, the eluents containing biotin-labelled antibody were mixed well, and added with 2% BSA and 0.1% ProClin 300, stored at 4 °C for a short time, or added with 50% glycerol and stored at −20 °C for long-term storage.

MFNPs-AEPMs-CLIA for the determination of cTnI

The schematic diagram of the MFNPs-AEPMs-CLIA on cTnI is shown in Fig. 1. Four antibodies recognized different epitopes on cTnI molecules were utilized. We used biotin-labelled anti-cTnI antibodies (MF4, 560) immobilized onto SA-MFNPs by means of a biotin streptavidin system as the capture antibody and the AEPM-labelled anti-cTnI antibodies (19C7, 810) as the detection antibody. Finally, 10 μL of SA-MFNPs, 50 μL of biotin-labeled antibodies (diluted by 200 times), 50 μL of sample, and 50 μL of AEPM-labeled antibodies (diluted by 1000 times) were added into the special detection tube one time. cTnI present in the sample and AEPM-labelled anti-cTnI antibodies bound to the anti-cTnI MNPs to form a sandwich complex in a constant-temperature incubator at 37 °C for 10 min. After incubation, the products were washed with TBST for three times, and the supernatant was discarded by magnetic separation before the constant-temperature trigger solutions at 45 °C were added. The luminous intensity and fluorescence intensity of each tube were detected immediately using a 6800 chemiluminescence analyser (Getein, Nanjing). The concentration of the sample was calculated automatically through the standard curve after luminous intensity (RLU) was corrected by the fluorescence signal (AU) on the instrument. The correction formula is as follows:

$$RLU = \frac{AU_1}{AU_0} \times RLU_1$$

RLU is the chemiluminescence intensity calculated after calibration; AU\(_1\) is the initial fluorescence intensity of the fluorescent magnetic particles; AU\(_0\) is the fluorescence intensity measured using a fluorescence detector; and RLU\(_1\) is the chemiluminescence intensity measured using the photomultiplier tube (PMT).

Meanwhile, to demonstrate the high sensitivity and excellent precision of the reagents described in this paper, we checked the commercial kits (Getein CLIA kit) for comparison applying the same antibodies and the same instruments without an amplification system and fluorescence correction.

Assessment of analytical performance

Limits of the assay and functional sensitivity. Testing to determine the limits of blank (LoB), detection (LoD), and quantification (LoQ) was performed following recommendations in the Clinical Laboratory Standards Institute (CLSI) guideline EP17-A2. LoB, LoD, and LoQ were analysed and compared on two reagents including the Getein kit and the proposed kit, and the experimental design listed below was selected to meet the minimum requirements following the CLSI guideline. Blank samples were made by immunoabsorption of individual patient serum samples to remove cTnI. LoB was assayed with one instrument system, five blank samples, and four replicate measurements per sample for five days, with a combination of 100 total blank measurements (5 days × 5 samples × 4 replicates). LoD was assayed with five low concentrations of cTnI samples taken from the LoB samples and ranged from LoB to 3-times LoB (4 duplicates over 5 days). The default values of α = β = 0.05 were used for the Type I and II error risks. LoB and LoD calculations are derived parametrically or nonparametrically as appropriate, using both concentration and raw RLU data.

The LoQ is defined preferentially in terms of a total error (TE) goal or concerning goals for both bias and precision. In general, bias often is best estimated through the recovery of standards or samples having an accepted reference value. Precision estimates for the LoQ application should reflect both repeatability and day-to-day variability. The cTnI accuracy goal is a TE of 20%, based upon the desirable TE specification from the biological variation database provided by Carmen Ricós.39 The LoQ sample panel consisted of nine low-cTnI positive serum samples with concentrations spanning the range of approximately one to five times greater than the estimated LoQ.

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concentration (0.50–10.00 pg mL\(^{-1}\)). The experimental design met the minimum requirements with one instrument system, nine low-cTnI positive serum pool samples, 20 testing days, two runs per day, and two replicates per run (total of 80 replicates per sample). The classical Westgard model was used to define the TE for this evaluation: \(\text{TE} = |\text{Bias}| + 2s\).

**Precision**

Precision testing was also performed on two reagents including the Getein kit and the proposed kit following the CLSI EP05-A3 guideline. A panel with two samples with different cTnI concentration levels ranging from 1.0 to 10 000 pg mL\(^{-1}\) was prepared using different patient serum samples. The protocol used a nested components-of-variance design with 20 testing days, two runs per testing day, and two replicate measurements per run (a \(20 \times 2 \times 2\) design) for each sample. (Total number of replicates: 80 per site, Level 1: 125.94 pg mL\(^{-1}\), Level 2: 1050.25 pg mL\(^{-1}\).)

**Evaluation of potentially interfering endogenous substances**

In the MFNPs-AEPMs-CLIA of cTnI, potential interference from endogenous substances is \(\leq 10\%\) at the levels tested. A study based on guidance from the CLSI EP7-A2 was performed for the high sensitive cTnI assay. Samples containing high concentrations of potentially interfering substance (total bilirubin, 50 mg dL\(^{-1}\); triglycerides, 5000 mg dL\(^{-1}\); hemoglobin, 800.0 mg dL\(^{-1}\); and total cholesterol, 4000 mg dL\(^{-1}\), and cTnI concentrations lower than the detection limit were prepared. These were added in fixed ratios (0 + 10, 1 + 9, 2 + 8, 3 + 7, 4 + 6, 5 + 5, 6 + 4, 7 + 3, 8 + 2, 9 + 1, 10 + 0) to two sample pools containing different cTnI concentrations (S1: 15 pg mL\(^{-1}\); S2: 500 pg mL\(^{-1}\)). In addition, diluting both the S1 and S2 pools (in the same fixed ratios mentioned above) with a sample pool with undetectable concentrations of cTnI allowed us to produce a set of control dilutions.

**Detection of cTnI in the clinical serum samples**

Clinical samples used in this study were obtained from The Second Hospital of Nanjing. Serum specimens should be centrifuged at an RCF of 3000g for 10 minutes. Centrifuged specimens with a lipid layer on the top must be transferred to a sample cup or secondary tube. Care must be taken to transfer only the clarified specimen without the lipemic material. cTnI of 181 clinical serum samples were determined using the proposed method and Getein commercial kit (the same antibodies labelled AE without P(NIPAM-co-MAA) microspheres, and magnetic nanoparticles without fluorescence), and the results were both compared with that obtained by the classical method (Abbott ARCHITECT i2000 analyser CLIA). Linear correlation analysis was performed. The regression equation and correlation coefficients were used to judge the correlation between the two methods. To determine the consistency, the Bland–Altman method was used for stochastic effect analysis on their differences, with the average of concentration determined by two methods as the \(X\)-axis, and error (the difference to average) as the \(Y\)-axis. Finally, the mean relative error and the 95% confidence interval were determined.

**Characterization**

The morphologies of the microspheres were characterized using scanning electron microscopy (SEM, JSM-5600LV, Japan). Fluorescence measurements were recorded on a fluorescence spectrophotometer (Getein, Nanjing, China). Chemiluminescence measurements were recorded on a photomultiplier tube (PMT) (Getein, Nanjing, China). The particle size distribution and \(\zeta\)-potential of magnetic fluorescent nanoparticles and AE-loaded microspheres were determined using a Zeta Potential Analyzer (Zeta Plus, Brookhaven, USA). More experimental details are given in the supplementary text.

**Statistical analysis**

Statistical analysis on a completely randomized design was conducted using the one-way analysis of variance (ANOVA) procedure, with SPSS 17.0 software, at a level of significance set at \(p = 0.05\). The Bland–Altman plot and Passing–Bablok regression analyses were performed with MedCalc Software (MedCalc, Mariakerke, Belgium). All data were presented as mean values with their standard deviations (mean ± S.D.). Differences were accepted as significant when \(p < 0.05\).

**Results and discussion**

**Characterization of microspheres**

The SEM images of the magnetic fluorescent nanoparticles are given in Fig. 2A, C, and AE-loaded microspheres in Fig. 2B and D which clearly show a discernible structure with an average size of \(\sim 1000\) nm and \(\sim 200\) nm. The particle size distribution confirmed the SEM characterization. The \(\bar{Z}\)-average particle sizes of the magnetic fluorescent nanoparticles and AE-loaded microspheres were 1064.4 nm and 178.4 nm respectively. Furthermore, the polydispersity of the magnetic fluorescent nanoparticles and AE-loaded microspheres was 0.051 and 0.031. A uniform and stable structure is the basis for the use of the microspheres for creating a CLIA detection method.

To compare the ratio of acridine ester labelled antibody between regular labelling method and labelling amplified by microsphere, the RLU of AE was statistically calculated at the same antibody concentration, as shown in Fig. 2F. The total RLU of the regular labelling method was 1295846, and that of the amplification labelling method was 12036865. The improved labelling method increased the AE-label antibody ratio by almost 10 times, which was crucial to improving the sensitivity of the detection reagent. The fluorescence intensities of blank and magnetic fluorescent nanoparticles were shown in Fig. 2E. With 490 nm excitation, blank magnetic microspheres excited no obvious fluorescence signal, indicating that there was no fluorescent material in the magnetic bead. However, the fluorescent microspheres prepared in this
work produced a strong fluorescence signal at 520 nm, indicating that the FITC/Rhodamine 110 has been loaded into the magnetic microspheres successfully. We also studied the stability of the AEPMs and MFNPs by performing measurements of the luminous intensity and fluorescence intensity using fresh microspheres and microspheres stored at room temperature for 1 week, 1 month, 2 months, and 3 months. As shown in Fig. 2G and H, there is a negligible difference in the signals of the fresh and stored sensors, indicating that AEPMs and MFNPs exhibit excellent stability at room temperature.

Limit of blank (LoB), limit of detection (LoD), and limit of quantitation (LoQ) measurement

As a standard biomarker for the diagnosis of heart attacks, high sensitivity is required for cTnI assay. In this work, LoB, LoD, and LoQ were calculated based on the Clinical Laboratory Standards Institute (CLSI) Guideline EP17-A2. The RLU of the negative sample was measured for 100 times. As rank positions are integers, the value corresponding to a rank position 95 was LoB. Table 1 contains the upper-rank values for the sorted blank measurement results and LoB was determined as 1.06 pg mL\(^{-1}\) for the Getein kit, and 0.097 pg mL\(^{-1}\) for this improved kit.

LoD values of the two reagents were determined to be 1.400 pg mL\(^{-1}\) and 0.116 pg mL\(^{-1}\) respectively according to the calculation shown in Table 2 giving the SD\(_L\) values based on the individual SDs for each low sample. The LoD estimate for each reagent kit was calculated following the equation: LoD = LoB + \(C_pSD_L\), using the reported LoB determined above (\(C_p\) was a multiplier to give the 95th percentile of a normal distribution, and the calculated result is \(C_p = 1.649\)).

The TE calculations are summarized in Table 3. Using 20% TE as the accuracy goal, the LoQ of each reagent kit was determined with Hybritech calibration. As shown in Fig. 3, the lowest sample concentration that met the accuracy goal for the Getein kit is 2.393 pg mL\(^{-1}\), and the improved kit was 0.606 pg mL\(^{-1}\).

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<td>(C_p)</td>
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<td>LoD</td>
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<tr>
<td>Low8</td>
<td>9.0</td>
<td>9.68%</td>
<td>3.89%</td>
</tr>
<tr>
<td>Low9</td>
<td>10.0</td>
<td>7.11%</td>
<td>3.62%</td>
</tr>
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</table>
Limits of detection and quantitation are critical and important for many parameters because the ultrasensitive techniques for protein detection play essential roles in promoting the early diagnosis of diseases, as in the case of troponin I. In these traditional immunological methods, the labeled antibodies that usually are linked with various labels, such as enzymes, illuminant dyes, and radioactive elements at a low ratio, are employed to specifically recognize the target proteins and indicate detection signals with competitive or sandwich mode. There is no doubt that the low ratio of signal label to antibody results in a limited detectable signal and relatively low sensitivity of these traditional immunoassays. Due to the strong signal intensity of AE in use of the amplification effect of AEPMs, the limits of the established method were about 10 times greater than that obtained using the AE-labeled anti-cTnI (a commercial cTnI chemiluminescence kit, Getein). The enhancement of the sensitivity achieved using P(NIPAM-co-MAA) microspheres could be attributed to a bigger number of AE capped within the microspheres (∼100 AE/one microspheres). However, in the conventional AE labeling antibody process, there were generally only ∼10 AE on each antibody. This proposed method made full use of the inner space of the microspheres, which could avoid the competition between the antibody and acridine ester on the surface of microspheres during the coupling process. In contrast, the conventional method only utilized the surface of the microsphere as the carrier to mark the signal amplification. Besides, the excellent labeling amplification can be realized by using temperature-sensitive gel particles because it can avoid the fact that the acridine ester cannot be excited due to the strict encapsulation. Compared with these routine labeling techniques, our proposed method has been demonstrated to be a more sensitive and powerful tool for the determination of cTnI.

**Precision**

We studied the intra- and inter-day for the improved CLIA in the presence of MFNPs and AEPMs as shown in Table 4. Two fluorescent materials with similar excitation and emission wavelengths were used as the internal standard to evaluate the improvement of precision. When using FITC as an internal standard, the intra-day variability ranged from 1.21 to 2.12% for various cTnI concentrations, while the inter-day variability was between 2.01 and 3.49%. There was no obvious distinction in the improvement of precision when rhodamine 110 was used as an internal standard instead of FITC. In this experiment, compared with the commercial immunoassay (Getein Kit, negative control) which only made use of conventional magnetic nanospheres, the precision had been significantly improved over 50%. The reason is that the relative variation ratio of the fluorescence intensity is used to correct the luminescent signal by the formula described above under the conditions that the fluorescent signal and the luminous signal do not interfere with each other in the detection. The deviation of the expected value may be caused by systematic random errors such as the sampling process, or the cleaning process of the magnetic bead immune complex, etc. In this series of processes, a significant improvement on the precision was achieved by means of a fluorescent signal as an internal standard to eliminate the instrument system error. The results indicate that the CLIA in the presence of MFNPs and AEPMs

can be applied as a new analytical method to quantify cTnI in the human serum with acceptable precision.

**Evaluation of potentially interfering endogenous substances**

Potentially interfering endogenous substances were evaluated to determine the impact on cTnI results. Samples with cTnI concentrations of 15 pg mL\(^{-1}\) and 500 pg mL\(^{-1}\) demonstrated interference within ± 10% for the potentially interfering substances listed in Table 5. No significant interferences were observed with icteric (bilirubin up to 45 mg dL\(^{-1}\)) or hemolyzed (hemoglobin up to 560 mg dL\(^{-1}\)) samples. Triglyceride concentrations at 3000 mg dL\(^{-1}\) and total cholesterol at 2000 mg dL\(^{-1}\) appeared to interfere with MFNPs-AEPMs-CLIA of cTnI measurements in one of the two samples tested S2. However, given that this difference is well within the imprecision of the assay, it could be considered not clinically relevant.

**Clinical sample analysis**

To evaluate the practical application of the MFNPs-AEPMs-based CLIA method for the cTnI assay, a total of 181 serum samples including 51 low-level concentration samples (<1 ng mL\(^{-1}\)), 54 median value samples (1–5 ng mL\(^{-1}\)), and 76 high-value samples (>5 ng mL\(^{-1}\)) were analysed by the developed method and Getein kit as a negative control. The results of the two groups were compared with the results from the Abbott kit assay respectively. Comparisons of both serum results were made based on the Bland–Altman plot, and Passing–Bablok regression analysis, as shown in Fig. 4. The correlation coefficient (\(R^2\)) for the regression line of the Passing–Bablok regression analyses of the two reagents were 0.9720 and 0.9883, indicating that the proposed method performed a better linear relationship than the Getein Kit. The mean relative differences (95% limits of agreement) were 0.37 and 0.25 from Bland–Altman plot results, revealing that the proposed method has a better consistency than the negative control with the Abbott Kit. The improved reagent kit can correct the measured value with the help of internal parameters, and less random fluctuation can effectively reduce the discretization of the linear fitting to improve the correlation with the control reagent, especially the lower value part with large random error. It can be concluded that the improved immuno-assy was demonstrated to offer good accuracy in clinical testing.

**Table 5** Testing of potential interference from endogenous substances using the MFNPs-AEPMs-CLIA cTnI assay

<table>
<thead>
<tr>
<th>Potentially interfering substance</th>
<th>Potentially interfering substance concentration</th>
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<tbody>
<tr>
<td>Total bilirubin</td>
<td>≤45.0 mg dL(^{-1})</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>≤2000.0 mg dL(^{-1})</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>≤560.0 mg dL(^{-1})</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>≤3000 mg dL(^{-1})</td>
</tr>
</tbody>
</table>

**Conclusions**

A high-sensitivity and excellent precision CLIA has been developed for the clinical determination of cTnI in the human serum, using the AE chemiluminescence system combined...
with P(NIPAM-co-MAA) microspheres, and fluorescent magnetic nanoparticles. This assay provides apparent advantages and shows great potential in the clinical diagnosis.

**Conflicts of interest**

There are no conflicts to declare.

**Acknowledgements**

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**References**


