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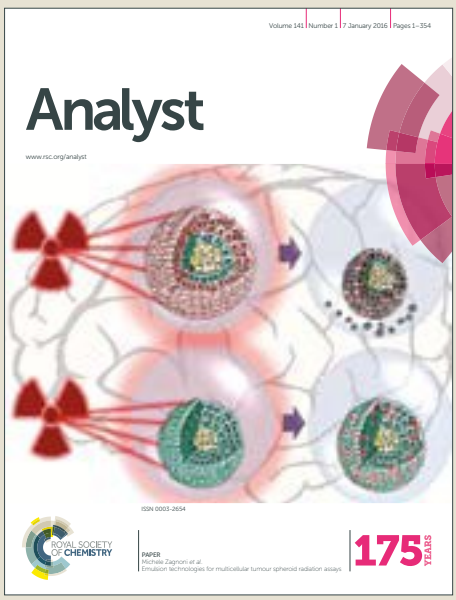

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DOI: 10.1039/C7AN01566F

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ARTICLE

Development of fluorescence capillary biosensor based on self-assembled AuNPs/LDH for micro-volume intracellular pyruvate

Received 00th January 2017,
Accepted 00th January 2017

DOI: 10.1039/x0xx00000x

www.rsc.org/

Ya-Xiong Luo[a], Qiao-Jing Li[b], Lang Zhou[b], Yong-Sheng Li[*b] and Xiu-Feng Gao[*a]

Abstract: A fluorescence capillary biosensor for quantifying micro-volume intracellular pyruvate (PA) was developed, in which AuNPs and lactate dehydrogenase(LDH) were modified on inner surface of the amination capillary (20 μL) via self-assembly technique. The PA concentration was quantified by the change value of fluorescence of NADH after sucking the mixture solution of sample and NADH into the biosensor. This study investigated these factors including protonation degree of amino on surface of capillary, the AuNPs concentration and time for self-assemble, activity concentration and time of LDH for self-assemble, the flow rate and acidity for LDH immobilization, pH, temperature and reaction time for the NADH/PA/LDH reaction system and so on. Under optimized determination condition, the linear response range of this biosensor for PA was 2.5-120 μmol/L, in which determination limit and detection limit were 2.5 and 0.75 μmol/L respectively. The biosensor could be reused more than 41 times when its relative standard deviation (RSD) was controlled at less than 1.5%. Under room temperature (approximately 25°C), the intracellular PA in erythrocyte of healthy person was measured with the biosensor and the gained PA content was 241.76 ± 68.05 μmol/L (n=8). The standard addition recovery was 95-106%. Employment for AuNPs in PA biosensor not only improved affinity of immobilized LDH to PA and itself stability, but also significantly enhanced service life of PA biosensor.

Introduction

Pyruvate (Pyruvic acid, PA), the end-product of glycolysis, is reduced to lactate under catalytic action of lactate dehydrogenase(LDH) for the cellular cytoplasm in anoxia; PA is ultimately destined for transport into mitochondria where it is oxidized to CO_2 and H_2O in sufficient oxygen.[1] However, in the tumor metabolism even if the oxygen is sufficient, the energy in the tumour metabolism is provided through glycolysis.[2] Therefore, the detection for intracellular PA in tumour also began to be paid close attention to.[3,4] Currently, various methods for the detection of PA had been reported including capillary electrophoresis (CE)[5-7] high-performance liquid chromatography (HPLC),[8-10] enzyme-fluorescence method.[4,11]

Xue et al. firstly reported the use of CE method for detection PA in erythrocytes.[5] A single red cell drawn into the capillary inlet with the help of the microscope, the capillary was immediately moved back into the running buffer (pH 8.5) containing glucose, fluorescein sodium (λex/em: 330/400nm) and Tris to make the cell lysis by glucose solution and then the separation for lysed PA began with adding voltage. Finally, the PA was quantified by detecting negative spike derived from the concentration decrease of local fluorescein in capillary based on the charge displacement between pyruvic acid radical and fluorescein with negative charge. The relative standard deviation (RSD) of the method was approximately 20%, and the calculated concentration for PA was 22.8-25.6 mmol/L. However, the accuracy for PA detection was influenced by some factors (e.g., glucose concentration, sucked amount of cell suspension and capillary shaking in operation). Soga et al. improved CE method by coating a cationic polymer (polybrene) in inner surface of capillary and using electrospray ionization mass spectrometry as detector to measure intracellular PA and anionic intermediates for *Bacillus subtilis*.[6] The response range for PA in the method was 5-100 μmol/L via using ammonium acetate solution (50 mmol/L, pH9.0) as separation electrolyte. Markuszewski et al. utilized cetyltrimetylammonium bromide instead of cationic polymer and UV photometer instead of detector above CE method to research PA assays in human red blood cells.[7] The method using 2, 6-pyridinedicarboxylic acid as a highly UV absorbing carrier electrolyte (pH12.3, 25kV) quantified PA by negative spike generated at 214nm, which was in the determination range of 50-400 μmol/L.

There were several reports on HPLC methods of determination for intracellular PA. Bhattacharya et al. used conductometer as detector of HPLC, Dionex IonPac AS11 cationic column as separation column and NaOH as mobile phase to investigate PA assays in the range of 1-500 μmol/L.[8] Due to the near retention time for high concentration of chloride in real sample and PA in the method, it was not possible to realize effective segregation and detection for PA

[a] West China School of Basic Medical Sciences & Forensic Medicine, Sichuan University, Chengdu 610041, China.
[b] School of Chemical Engineering, Sichuan University, Chengdu 610065, China.
* Response Author E-mail: li-yongsheng@scu.edu.cn； xiufengg@163.com

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in cell sample. Luo et al. utilized mass spectrometry as detector of HPLC, C18 as separation column and tributylamine/ acetic acid/ methanol as mobile phase to study the content of intracellular PA for *Escherichia coli*.[9] The response range for PA (0.5-25 μmol/L), relative standard deviation (RSD≤3.6%) and analysis time (approximately 70min/sample) were obtained. Ogasawara et al. researched the content of prelabeling intraerythrocyte PA using 1,2-diamino-4,5-methylenedioxybenzene with an excitation wavelength of 367nm and an emission wavelength of 446nm by using fluorophotometer as detector of HPLC, Merck LiChrosphere RP-18(e) as separation column and PBS buffer with acetonitrile as mobile phase.[10] The linear response for PA in the method was produced with concentration in the range 1-500 μmol/L and the concentration of prelabeling PA in erythrocyte was 430±10 μmol/L. Its analysis rate was 2 sample / h.

Enzyme-fluorescence method was also used to determine the concentration of PA. Tormey et al. firstly used the oxidation-reduction reaction of PA with NADH catalyzed by LDH to form the non-fluorescent NAD^+, and then investigated the alteration of PA concentration for mammalian cells by detecting the fluorescent decrease of NADH (λex/em: 340/460nm).[4] The obtained the concentration for PA were 138±3.1 μmol/L for fibroblasts; 60.4 ± 4.6μmol/L for CAMA-I human breast adenocarcinoma; 82.4 ± 5.2μmol/L for SK-BR-2-III human breast adenocarcinoma; 149.9 ± 8.0μmol/L for P815 cells; and 87.9 ± 4.0μmol/L for the L929 mouse connective tissue cell line. The detection limit and recovery for the method were respectively 0.84 μmol/L and 97±5%. Zhu et al. indirectly quantified intracellular PA in leukemia cell based detecting the produced fluorescent resorufin (λex/λem: 535/590nm) in the reaction of H_2O_2/Amplex Red catalyzed by horseradish peroxidase after pyruvate oxidase catalyzing the reaction of PA/ dissolved oxygen to generate H_2O_2.[11] The measuring range for the method was 0.05-2 μmol/ well and the detection limit was 5 nmol/L. Its results showed that the concentrations of PA were 201.0±20.7 μmol/L for leukemia cell and 134.0±26.2 μmol/L for leukemia cell treated with L-phenylalanine.

Fluorescent sensors have attracted particular attention due to their advantages such as low cost, operational simplicity, high sensitivity and selectivity[12-19]. Fluorescence capillary analysis (FCA) [20], a novel micro-volume detection method, utilizes a common medical glass capillary instead of traditional fluorescence test tank and micro-volume fluorescent pool. The glass capillary in the FCA is not only a sample container and a reactor container, but also is an immobilized carrier for enzyme, gene and reagent. Thus, FCA method, not only significantly reducing the waste discharge of chemistry, and but also really driving fluorospectrophotometry into practicability or universalness, really realizes microanalysis in micro-sample. At present, the application for FCA method had been reported including ethanol in wine[21], PA in serum[22], sulfated bile acid in urine[23] and target nucleotide[24].

Furthermore, Zhou et al. firstly utilized FCA method in the free LDH/NADH/PA reaction system to rudimentary study the determination for intracellular PA[25]. Then, our previous work[26] developed a novel Fluorescent capillary sensors for detection of lactate based on immobilized enzyme, however, the immobilization of LDH on the inner surface of capillary with glutaraldehyde as a cross-link agent had some questions (e.g., complicated preparation process, strict condition and not high enzyme activity which cause low sensitivity that cannot satisfy for determination of intracellular PA). Gold nanoparticle has most popular due to its controlled size, super-large specific surface area and characteristic optical property, catalytic activity, surface like charges and widely use in electrochemical[27-29] and enzyme immobilization research[30] Therefore, in this work using AuNPs with negative charge instead of glutaraldehyde, AuNPs and LDH were immobilized in the inner surface of capillary by self-assembly technique[31, 32] to develop a novel fluorescence capillary biosensor (PA-FCABS) for application in the PA determination of human red cells.

Experimental methods

Chemicals and reagents

The main instruments and materials in the study including: RF-5301PC fluorophotometer (Shimadzu Co., Japan), Tecnai G_2 F_{20} transmission electron microscope (FEI, Hillsboro, USA), UV-1800 PC type UV-vis spectrophotometer (Meipuda, Shanghai, China), Dry multi heater (Aosheng, Hangzhou, China), TGW-12 supercentrifuge(Yingtai, Changsha, China), Medical glass capillaries(45mm X 0.90mm), self-made capillary holder (13mm X 13mm X 43mm), glass slice (40mm X 12mm X 1mm), vacuum blood collection tube (containing K_2EDTA)

The main reagents in this experiment including: LDH (EC 1.1.1.27, 9868 kU/L, Amersco, Solon, US), reduced form of nicotinamide-adenine dinucleotid(NADH), oxidized form of nicotinamide-adenine dinucleotid (NAD^+) and PA (Sanland, Amoy, China). trisodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), Tris and glutaraldehyde (Kelong, Chengdu, China), $HAuCl_4 \cdot 4H_2O$ (Maisike, Tianjin, China), 3-aminopropyl triethoxysilane (APTES, jianghan, Jingzhou, China), pyruvate (Sanland, Amoy, China). All used reagents were of analytical-reagent grade. Pure water (1.0 μS/cm) was used in all experiment. Blood samples were obtained from 8 healthy volunteers who were fasting for 12 h. The medical ethics committee of sichuan university reviewed and approved the informed consent forms provided by all participants according to ethical requirements.

Preparation of reagent Solutions

120 kU/L LDH: LDH solution (9868 kU L^{-1}) was exactly taken 60 μL in a 5-mL calibrated flask, and diluted to the mark with 50 mmol/L PBS buffer (pH7.5).

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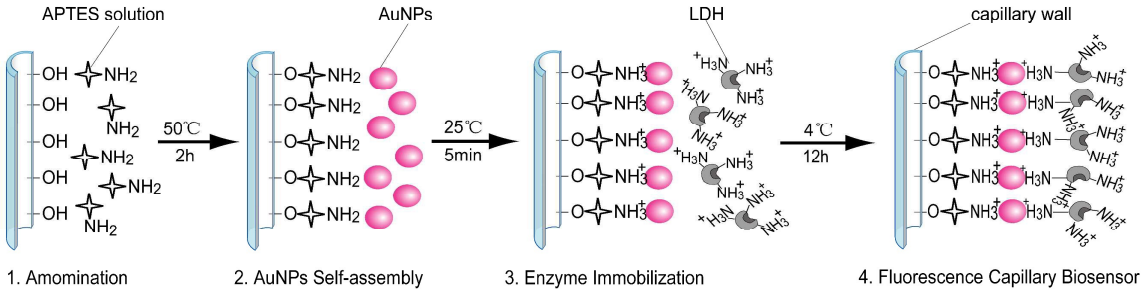

Fig. 1 Schematic of the fabrication for PA-FCABS.

2.0 mmol/L NADH stock solution: 7.64 mg of NADH was dissolved and diluted to the mark in a 5-mL calibrated flask with 50 mmol /L PBS buffer (pH7.5).

0.12 mmol/L NADH standard solution: 120μL NADH stock solution was exactly taken and diluted to 2.0 mL with 50 mmol/L PBS buffer (pH7.5)

2.0 mmol/L PA stock solution: This solution was prepared by dissolving 2.20 mg of PA in 5-mL PBS buffer (50 mmol /L, pH7.5)

0.12 mmol/L PA standard solution: 120μL NADH stock solution was exactly taken and diluted to 2.0 mL with 50 mmol/L PBS buffer (pH7.5)

Erythrocytes lysate buffer:[25] This solution was prepared by dissolved 80.20 g of NH_4Cl (1.5 mol /L), 8.40 g of $NaHCO_3$ (100 mmol/L) and 3.70 g of Na_2EDTA (10 mmol/L) with 900 mL water, then adjusted pH to 7.4, and finally diluted to 1.0L by adding water; the erythrocytes lysate was diluted 10 times before using.

Above overall reagent solutions were stored at 4°C, taken out at room temperature before using for 30min.

Fabrication of AuNPs

AuNPs collosol were prepared by the method of citrate reduction of gold (III) chloride solution.[33] Operational process were that 3mL of trisodium citrate solution (38.8 mmol/L) was quickly added to 100mL chloroauric acid solution (1.0 mmol/L) at boiling temperature. After continuously heated and stirred for 15min, a color change of a yellowish to wine red for the solution was observed. Then the solution with wine red was cooled to room temperature in continuously stirring after removing heat source. The AuNPs collosol was filtrated with 0.5μm of membrane, enclosed into brown bottle and stored at 4 °C.

Procedure of fabrication for PA-FCABS

The literature reported a biosensor for determining lactic acid based on LDH immobilized on inner surface of capillary by using glutaraldehyde as cross-linking agent.[26] However, the enzyme loading and sensitivity for the method was lower. Hence, to resolve above question, utilizing AuNPs instead of glutaraldehyde, the study fabricated a novel PA biosensor by self-assembly. The schematic for the fabrication was showed in Fig. 1.

1) Pretreated capillary and amination capillary were identical with literature.[34]

2) AuNPs self-assembly: Firstly, the amination capillary was dipped in hydrochloric acid (pH1.0) for 10min to protonate amino of APTES in surface of capillary. Then hydrochloric acid solution in capillary was blown out and AuNPs colloidal solution with negative charge (7.0 nmol/L) was sucked into protonation capillary, the internal fluid in capillary blown out after self-assembly for 5min at room temperature.

3) LDH immobilization: The capillary with AuNPs was dipped in LDH solution circularly flowed at 4 °C, and internal fluid in capillary was blown out after 12h. Finally, the PA-FCABS fabricated was stored in 50 mmol/L of PBS (pH7.5) at 4 °C.

The calculation of self-assembly quantity of enzyme in the PA-FCABS

Based on the method of fluorescence capillary analysis for LDH activity[35], the study utilized the enzyme activity units retained per unit surface area denoting the self-assembly quantity of enzyme (Qs) in inner surface of PA-FCABS:

$$Q_S = \frac{\Delta F}{\Delta t} \times \frac{d}{4K_{NADH}} \quad (1)$$

Where Δt is the time interval of reaction for PA with NADH; ΔF is the fluorescence decrease of NADH at 460nm; d is the inner diameter(0.090cm) of PA-FCABS; K_{NADH} presents the proportional coefficient of fluorescence intensity with concentration of NADH ($\Delta F/\Delta c_{NADH}$, L /mol).

Principle and procedures of determination for PA

In presence of the mix solution of PA sample with NADH at pH7.5, LDH in PA-FCABS catalyzed PA to form lactic acid, and simultaneously NADH converted into NAD^+, the content of PA indirectly quantified by detecting the fluorescent decrease (ΔF) of NADH.

Procedures of determination for PA: At room temperature, PA-FCABS sucking the mixture (20μL) with same volume of sample and NADH regent was inserted in the capillary holder of fluorophotometer to detecting a fluorescent blank (F_1). Then, the PA-FCABS was taken out from the holder and inserted in the capillary holder again after 5min to obtain the fluorescence intensity (F_2). Finally, the content of PA was

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calculated by a fluorescent decrease in accordance with the fluorescent difference value twice ($\Delta F = F_1 - F_2$).

Results and Discussion

Investigation of AuNPs modification and LDH self-assembly

AuNPs prepared by the method of citrate reduction of gold (III) chloride solution was characterized by transmission electron microscope (TEM). Obtained result was shown in Fig. 2a, indicating that the synthesized AuNPs had an average diameter of 13nm with sphere, uniform dispersity. According to the atomic weight (196.96) and density of gold (19320g/L), the volume of Au atom could be calculated. Next, the volume of gold nanoparticle was divided by the volume of Au atom, which equates to 67951 atoms per gold nanoparticle. Finally, AuNPs concentration estimated at approximately 14nmol/L was obtained by dividing the 67951 atoms per gold nanoparticle into the total number of gold atom in 1.0 mmol/L of chloroauric acid.

To conveniently observe the self-assembly effects for AuNPs and LDH on inner surface of capillary, in here, a glass slice which was almost the, same as glass capillary in matter was used. The spectral scans of every fabrication procedures in accordance with fabrication for PA-FCABS were carried out by the ultraviolet visible spectrophotometer. As shown in Fig. 2b, free LDH had a maximum absorption at 256nm, and AuNPs collosol had a specific absorbing peak at 518nm which was identical with literature reported.[36] In the presence of AuNPs immobilized on the surface of glass slice, a maximum absorption peak at 518nm was also observed. Subsequently, while AuNPs and LDH were simultaneously immobilized in the surface of glass slice, there were two maximum absorption peaks at 256nm and 518nm, indicating that all of AuNPs and LDH were self-assembled indeed on the surface of glass slice, in addition, indirectly confirming that AuNPs and LDH were also successfully self-assembled in the inner surface of capillary according to the process operation of fabrication for PA-FCABS.

Optimization of fabrication conditions for PA-FCABS

Effect of the protonation degree for APTES on Qs: In order to investigate the effect of protonation degree for APTES on Qs in PA-FCABS, the capillaries modified by APTES were respectively dipped into the ultrapure water with different acidity (pH0.2, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0) adjusted by hydrochloric acid, and then the self-assembly of AuNPs (10min, 24 °C) and LDH (24h, 4 °C) were gradually carried out to form multiple PA-FCABS after protonation for 10min. After sucking reaction mixture made up of 1mmol/L PA+1mmol/L NADH into PA-FCABS, the detected ΔF was taken into the equation(1) to calculate corresponding Qs. Finally the optimal protonation acidity of APTES was judged according to the value of Qs. From the relationship between pH value and Qs (Fig. 3a), we found that Qs was increased with the increase of pH in the range of pH 0.2-1.0. However, Qs was decreased with the increase of pH in the range of pH 1.0-5.0. Hence,

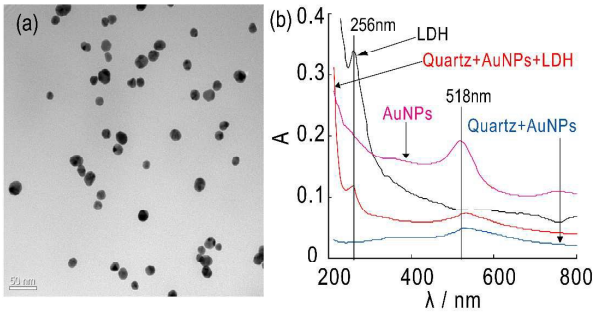

Fig. 2 TEM micrograph of AuNPs (a) and characterization of the fabricated PA biosensor with absorption spectrum (b).

pH1.0 of acidity for hydrochloric acid solution was selected to adjust the amino protonation of APTES. It illustrates that within the scope of certain acidity, the amino protonation of APTES on inner surface of capillary is increased with the increase of pH value in soak solution of capillary, or in other words, increasing the quantity of amino with positive charge. Thus the increase absorptive capacity of AuNPs with negative charge causes the increase of self-assembly quantity for LDH finally. Whereas, with the pH value in soak solution of capillary decreased, the protonation degree of amino for APTES in inner surface of capillary is decreased and the absorptive capacity of AuNPs also is decreased, resulting in the decrease of the self-assembly quantity of LDH. On the other hand, it also confirms that the interaction between AuNPs and amino of APTES is electrostatic interaction.

Effect of the modification time of AuNPs on Qs: Under the same test conditions as above-mentioned, AuNPs solution (14nmol/L) was inhaled into capillary treated by amination and protonation, in which AuNPs were retained for different time. Then the self-assembly of LDH was performed to fabricate PA-FCABS. The optimal modification time of AuNPs was judged by the value of Qs calculated by detecting ΔF of reaction mixture in PA-FCABS. Obtained results were shown in Fig. 3b, indicating that the Q_s value of PA-FCABS was increased with the increase of the modification time for AuNPs. At the modification time over 5min, the Qs value was not variable. Its reason is that the interaction between amino with positive charge for APTES in the inner surface of capillary and citrate with negative charge in surface of AuNPs is electrostatic adsorption which is a fast reaction, resulting in the adsorption saturation reached up to rapidly. Thus the modification time of AuNPs was selected to be 5min finally.

Effect of AuNPs concentration on Q_s: Under the same test conditions as above-mentioned, the effect of AuNPs concentration on Q_s was investigated in the concentration range of 0.4-14 nmol/L while modification time of AuNPs was fixed for 5min. As shown in Fig. 3c, obtained experiment results indicated that the Q_s value was increased when AuNPs concentration was increased in the range of 0.4-7.0 nmol/L, or was not so significant at AuNPs concentration exceeding 7.0 nmol/L. The results explain that the high concentration of AuNPs causes more absorbed AuNPs in inner surface of

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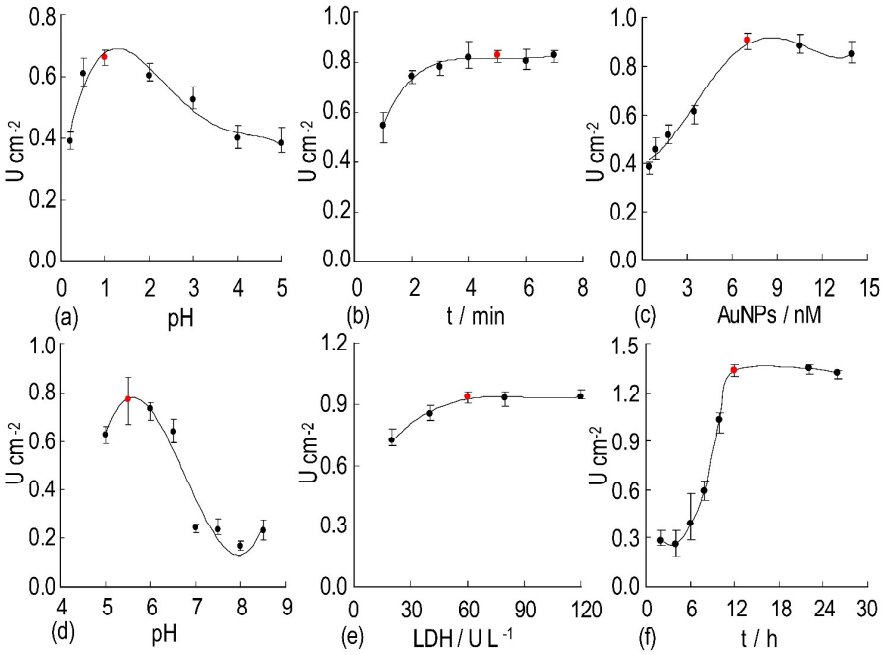

Fig. 3 Effects of the protonation of amino group of APTES (a), modification time of AuNPs (b), AuNPs concentration (c) and pH (d), active concentration (e) in enzyme solution, immobilization time of LDH (f) on Qs.

amination capillary, and more LDH are immobilized by electrostatic absorption up to the saturation of self-assembly. Hence, 7.0 nmol/L of AuNPs concentration was selected to be used in all subsequent experiments.

Effect of pH for LDH solution on Q_s: Under the same test conditions as above-mentioned, the effect of the acidity for LDH solution on self-assembly quantity of enzyme was investigated in the presence of the identical operation method. Fig. 3d showed that in the range of pH4.5-8.5, the value of Q_s was firstly increased and then keenly decreased. The value of Q_s reached to the maximum at pH5.5. It is attributed to the increase of amino with positive charge in LDH after protonation when pH value of LDH solution is lower, which is beneficial to electrostatic adsorption between LDH and AuNPs with negative charge. On the contrary, with pH of LDH solution increased, the deprotonation degree of amino for LDH and the ionization degree of carboxy group are increased, which will derive the increase of negative charge for enzyme molecule and produce the enhancement of electrostatic repulsion between LDH and AuNPs with negative charge and further occur the decline of the self-assembly quantity of LDH. Furthermore, excessive low pH or excessive high acidity for LDH solution may destroy the conformation of LDH and cause inactivation of LDH. Hence, the optimum pH of enzyme solution applied for the self-assembly of LDH was chosen to be 5.5.

Effect of active concentration for LDH on Q_s: Fixing above selected experiment conditions, the effect of active concentration on Q_s was investigated in the range of 20-120 kU/L for LDH, which was based on the identical operation method. Obtained results shown in Fig. 3e presented that the value of Q_s was gradually increased when the active concentration of the LDH was 20-60 kU/L, but the value of Q_s did not change with the increase of active concentration for LDH at the active concentration exceeding 60 kU/L. Finally, the active concentration of LDH for immobilization was selected to be 60 kU/L.

Due to the dynamic method excelling the static method for enzyme immobilization[37], thus the dynamic method was chosen in the experiment. Then the circulation rate of LDH solution was studied in the range of 0-0.56 ml/min (inner diameter of pump tubing: 0.5mm). The experiment results showed that the value of Q_s was highest at flow rate for 0.17 ml/min. So, the circulation rate of enzyme solution was chosen to be 0.17 mL/min.

The effect of self-assembly time for LDH on Q_s: Fixing above experiment conditions, the effect of contact time between LDH and capillary on Q_s was investigated in the presence of the identical operation method. Fig. 3f showed that Qs value was rapidly increased in the contact time for 2-12h (4 ^{o}C), but the self-assembly quantity of LDH did not change at the contact time over 12h. These results illustrate that the combination process of LDH with AuNPs in inner surface of capillary is slow. The reason for that is because LDH, compared with AuNPs, is biomacromolecule being of a smaller kinetic energy, furthermore containing a large number of carboxylate radical with negative charge rejecting $AuNPs^{-}$. Thus, the self-assembly time for LDH was selected to be 12h.

Michaelis constant of immobilized LDH in PA-FCABS

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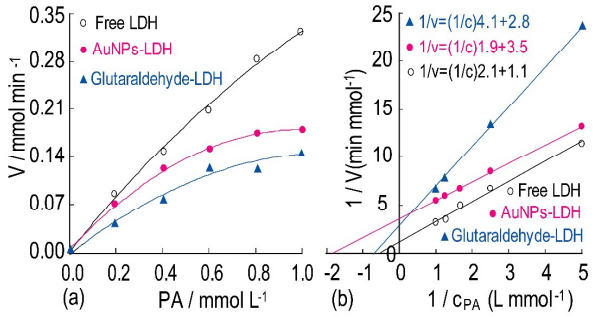

Fig. 4 Effects of concentration for PA on enzyme of PA-FCABS (a) and Lineweaver-Burk curves (b).

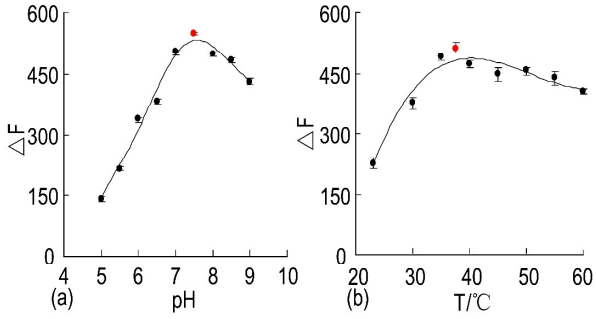

Fig. 5 Effects of pH (a) and temperature (b) in NADH/PA system on sensitivity for PA-FCABS.

To investigate the AuNPs | LDH layer affinity for the substrate in PA-FCABS, in the experiment michaelis constant of AuNPs | LDH layer in PA-FCABS was detected via using PA as the substrate[38] and respectively compared with free LDH and immobilized LDH by glutaraldehyde at the same test condition.

Operation process: the mixtures containing different concentrations of PA and the same concentration of 1.0 mmol/L NADH as reaction solutions were respectively inhaled into PA-FCABS. Next, the fluorescence decrease (ΔF) of reaction solution in biosensor was detected in the condition of 2 min for reaction time and 23.5 °C for reaction temperature and then put into the linear equation of ΔF-c_{NADH} (ΔF = 776.03 c_{NADH} + 2.35, r = 0.9983) to obtain the corresponding decrease of c_{NADH} in reaction solution, the velocity (v) of PA/NADH system catalyzed by LDH in the PA-FCABS indirectly calculated out finally. The relevant results were showed in Fig. 4a. Finally, Lineweaver-Burk curve[39] was obtained by using the reciprocal of PA concentration ($1/c_{PA}$) as x-coordinate and the reciprocal of reaction velocity ($1/v$) as y-coordinate (Fig. 4b). Analyzing and calculating for the data in the curve, the obtained apparent michaelis constants (K_m) for AuNPs | LDH layer, free LDH and glutaraldehyde | LDH layer were respectively 0.55, 1.90, 1.46 mmol/L. Significantly, the value of Km for AuNPs | LDH layer in PA-FCABS is much less than the value of K_m for free LDH and glutaraldehyde | LDH layer, which indicates that in PA-FCABS the AuNPs | LDH layer affinity for PA substrate is maximum.

Effect of conditions of NADH/PA system on sensitivity of PA-FCABS

Effect of pH for reaction system: Under the catalytic action of LDH, pH of reaction solution affects the reaction direction between PA and NADH.[40] Hence the effect of pH on determination PA via PA-FCABS was investigated. At 25°C of reaction temperature, 1.0 mmol/L of NADH concentration, 5 min of reaction time, 3X10nm of pass-band width and 1.0 mmol/L of PA as test sample, the effects of the acidity of reaction system adjusted by using different pH of PBS buffer solution (0.05mol/L) were obtained as Fig. 5a. Results showed that fluorescence intensity from PA-FCABS was the maximum at pH7.5 of reaction system. So, the pH of PBS buffer solution (0.05mol/L) adjusting the reaction acidity of LDH/NADH/PA system was chosen for 7.5.

Effect of temperature for reaction system: Fixing above selected experiment conditions, the effect of temperature of reaction system on determination PA by PA-FCABS was investigated in the presence of the identical operation method. According to Fig. 5b, we found that the value of ΔF was increased with the increase of temperature, and but gradually decreased at the temperature over 37.5 °C. So the optimum temperature for determination PA by PA-FCABS was 37.5 °C.

The stability of PA-FCABS

The thermostability of PA-FCABS: To investigate the thermostability of immobilized LDH in PA-FCABS and be compared with free LDH, the PA-FCABS and the capillary inhaling free LDH were together put in the thermostat at 25 °C and 37.5 °C respectively to detect activity of LDH at regular intervals. Examination results in Fig. 6a revealed that the relative activity of LDH for free LDH and immobilized LDH of PA-FCABS were 70% and 86% respectively after 14h according to the curve tendency at 25 °C and the relative activity of LDH for free LDH, and immobilized LDH of PA-FCABS were 70% and 86% respectively after 10h at 37.5 °C. The results above mentioned illustrate that the thermostability of self-assembly AuNPs | LDH layer for PA-FCABS is great.

The intraday stability of PA-FCABS: At the optimum experiment condition and 25 °C of reaction temperature, the intraday stability of PA-FCABS was studied by repeatedly measuring ΔF after inhaling reaction solution of 100 μmol/LPA+120 μmol/L NADH into PA-FCABS. As shown in Fig. 6b, the reuse times of PA-FCABS were 41 when ΔF was 520.8 ±7.6 and RSD was controlled below 1.5%. The interday stability of PA-FCABS: To observe efficacious storage time of PA-FCABS stored at approximately 4 °C, the ΔF`s variation of the PA/NADH mixture in four PA-FCABS was discontinuously examined once a week. The curve in Fig. 6c showed that the relative standard deviation of ΔF for PA-FCABS was less than 1.5% within 45 days, which indicates that the interday stability of PA-FCABS is essentially stable.

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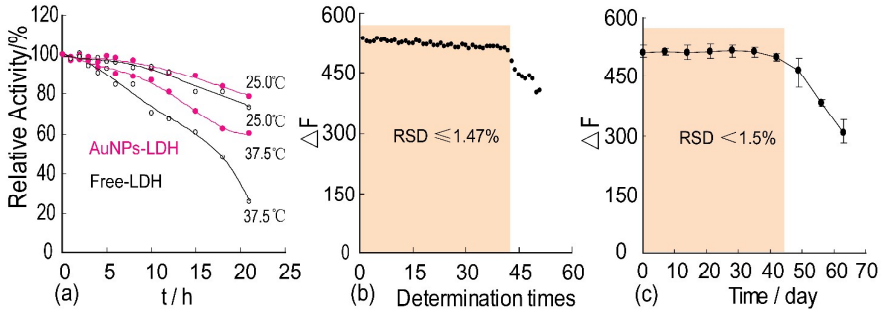

Fig. 6 The thermo-stability (a), intraday stability (b) and interday stability stability (c) of PA-FCABS.

The response time and linear range of PA-FCABS

The response time of PA-FCABS: The fluorescent response time of PA-FCABS was investigated by inhaling equal volume mixture of PA standard solution (20, 120 μmol/L) and NADH regent (1.0 mmol/L) into PA-FCABS. Obtained results were seen in Fig. 7a. It could be seen that the response signal for 20 μmol/L of PA standard sample reached up to response balance at 2min, and the response signal for 120 μmol/L of PA standard sample reached up to response balance at 5min. Hence, in consideration of response time for high concentration PA, the detection time of PA-FCABS was selected to be 5min. Furthermore, at difference temperature the fluorescent signal reaching up to balance was not significant, which indicates that the fast determination of low concentration PA was completed under room temperature. Although the optimum temperature was 37.5°C, in order to simplify the experiment operation, the reaction temperature also was chosen for 25 °C because of the sensitivity been reached up the requirement.

The linear range of PA-FCABS: Under the condition (25 °C, pH7.5, 120 μmol/L NADH) above selected, the reaction solution containing different concentration of PA was inhaled into the same PA-FCABS to perform the fluorescence determination, and then the PA standard curve shown in Fig. 7b was fabricated by PA concentration as x-coordinate and ΔF as y-coordinate. It could be seen that the linear correlation ($\Delta F = 5.27\ c_{PA} - 0.60$, $r = 0.9993$) for PA concentration with ΔF was great in the range of 2.5-120 μmol/L.

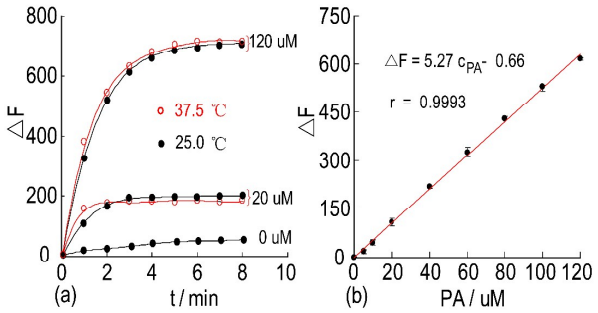

Fig. 7 The reaction time (a) and calibration curves (b) for PA determination with PA-FCABS.

The precision of PA-FCABS: A mixed solution with equal volume of PA (20 μmol/L) and NADH (120 μmol/L) used as the test sample was inhaled into PA-FCABS, and then the precision of determination PA for PA-FCABS was investigated by repeatedly detecting fluorescent signal of test sample in PA-FCABS. The obtained ΔF was 106.18 ± 1.32 and RSD calculated was less than 1.25% (n = 11), explaining that the repeatability of the method is great. In addition, Obtained detection limit (c_L) of PA-FCABS was 0.75 μmol/L (c_L = 3S/k, S =1.32ΔF, k = 5.27ΔF L/μmol) and determination limit (10S/k) was 2.50 μmol/L.

The interference of coexistent matter

Due to the interferential influence of various ions on determination of the object [41-50], the effects of intracellular coexistent matters on determination PA with PA-FCABS also were investigated. The mixture of 100 μmol/L PA+120 μmol/L NADH by adding the different concentration coexistent matters including NH_4Cl, $AlCl_3$, $MgSO_4$, KCl, $MnSO_4$, NAD^+, L-glutamic acid, glycine, ascorbic acid and glucose was inhaled into PA-FCABS, and then the PA concentration and recovery were calculated according to the response signal of detecting mixture. Obtained results were showed in Table 1, indicating that the coexistent matters in the range of added substances do not interfere for determining PA with the PA-FCABS.

Determination intracellular PA

Intracellular pyruvate extraction

In this paper, human erythrocytes were used as samples to determine the intracellular PA. The preparation and extraction of erythrocyte were done according to Zhou et al.[17] Blood (2.0 mL) was collected by venipuncture into anticoagulative tube containing 3.0 mg K_2EDTA, 4.0 mg NaF and 4.0 mg iodoacetic acid and then was stored on ice bath. Before using, anticoagulation for 1mL was centrifuged at 16000 rpm for 1 min at room temperature to remove plasma. Next, 100 μL of blood cells were transferred to a clean tube, suspended in 900 μL of erythrocytes lysate buffer to stand for 2min. That way obtained erythrocyte suspension was centrifuged at 16000 rpm for 1 min and then the supernatant

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Table 1 The tolerance molar ratios of co-existing materials for the determination of PA

Component	Added matter	Added conc. / mmol L^{-1}	Normal conc.(serum) / μmol L^{-1}	Recovery / %
Mg^{2+}	$MgSO_4$	0-7.5	800-1200	95.9-97.8
NH_4^+	NH_4Cl	0-20.0	0-20000	97.4-100.3
Mn^{2+}	$MnSO_4$	0-5.0	73-255	97.3-99.5
Al^{3+}	$AlCl_3$	0-2.5	0.01-0.22	98.2-99.5
K^+	KCl	0-25.0	3500-5300	100.5-103.1
Ascorbic acid	Ascorbic acid	0-9.0	30-120	98.5-100.8
L-glutamic acid	L-glutamic acid	0-15.0	—	102.7-04.5
Glucose	Glucose	0-25.0	3600-6100	96.2-98.3
Glycine	Glycine	0-15.0	6.0-30.0	95.3-99.3
NAD^+	NAD^+	0-5.0	—	94.9-101.3

was boiled for 5 min to degenerate protein. After centrifugation at 16000 rpm for 1 min, the supernatant was withdrawn and stored at 4 °C. The finally obtained supernatant was seen as sample of red blood cell lysate and its PA was stable in 5 days.

Determination of intracellular PA in human erythrocyte

PA-FCABS was used to perform the determination PA of red cell samples under selected experimental conditions, and the obtained results were presented in Table S1. The data of table showed that the PA content for eight red cell lysates was in the range of 16.29-34.49 μmol/L, and the PA content for human erythrocytes calculated was 241.76 ± 68.05 μmol/L. Each sample was repeatedly determined for 11times, resulting in RSD <5.1%.

Recovery tests

In addition, to further confirm accuracy of the PA-FCABS method to specifically detect the PA content in samples, four PA standard solutions were added to No. 1 - No. 8 sample solutions respectively, which was mixed with standards by 1+1 ratio, and then the recovery test was carried out. The experimental results were listed in Table S2. The recovery data were in the range of 95-106%, further, proving that the results of the PA-FCABS method for determination of intracellular PA in erythrocyte were accurate and reliable.

Conclusions

Based on self-assembly, the preparation of PA-FCABS by modifying AuNPs and LDH into medical glass capillary not only enhanced immobilization efficiency, catalyzed activity of LDH, and shorten test time of PA, but also resolved these current questions for the method of determination intracellular PA including low sensitivity and accuracy, high cost of test instrument, realized the goal of high sensitivity determination of PA in micro-volume cell sample. The advantages of the PA-FCABS method were high affinity of LDH layer to substrate PA, great thermal stability, long service life, high selectivity, great repeatability, and well linear correlation for PA concentration with ΔF. The proposed method was expected to be applied in clinical disease diagnosis and the studies for energy metabolism of a variety of normal cells, cancer cell.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We would like to thank volunteers who offered the blood samples and their support.

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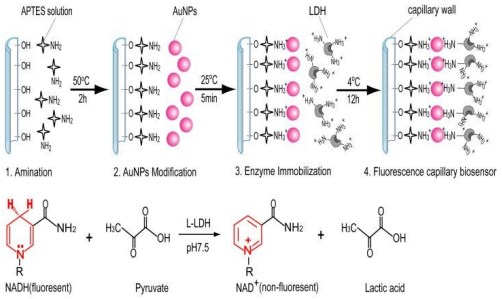

A high sensitive fluorescence capillary biosensor based on self-assembled AuNPs/LDH for micro-volume intracellular pyruvate