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A gold nanoparticle-mediated enzyme bioreactor for inhibitor screening by capillary electrophoresis

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ABSTRACT

A facile protocol to prepare highly effective and durable in-line enzyme bioreactors inside capillary electrophoresis (CE) columns was developed. To demonstrate the methodology, L-glutamic dehydrogenase (GLDH) was selected as the model enzyme. GLDH was first immobilized onto 38-nm-diameter gold nanoparticles (GNPs), and the functionalized GNPs were then assembled on the inner wall at the inlet end of the CE capillary treated with polyethyleneimine (PEI), producing an in-line GLDH bioreactor. Compared with a GLDH bioreactor prepared by immobilizing GLDH directly on PEI-treated capillary, the GNP-mediated bioreactor showed a higher enzymatic activity and a much better stability. The in-capillary enzyme bioreactor was proven to be very useful for screening of GLDH inhibitors deploying the GLDH-catalyzed α-ketoglutaric acid reaction. The screening assay was preliminarily validated by using a known GLDH inhibitor, namely perphenazine. A Z' factor value of 0.95 ($n = 10$) was obtained, indicating that the screening results were highly reliable. Screening of GLDH inhibitors present in medicinal plant extracts by the proposed method was demonstrated. The inhibition percentages were found to be 53% for *Radix scutellariae*, 45% for *Radix codonopsis*, 37% for *Radix paeoniae alba*, and 0% for the other 22 extracts tested at a concentration of 0.6 mg extract/ml.

Enzyme inhibitor screening is an effective approach to identify drug leads because many enzymes are involved in regulatory cellular processes and, thus, become therapeutic drug targets [1–3]. High-throughput screening (HTS)[1] is the most commonly used method for screening of enzyme inhibitors [4–6]. It is based on colorimetric or fluorometric measurements carried out on multiwell microplates. It works perfectly with large libraries of pure compounds but sometimes is not applicable to assay complicated samples such as extracts of medicinal plants that may contain many ultraviolet (UV)-absorbing or fluorescent compounds [7,8]. Over the past years, the search for enzyme inhibitors present in medicinal plants has been one of the major interests in drug discovery and development [9–11]. In fact, many new drugs developed between 1981 and 2002, particularly for treating cancer and infectious diseases, originated from natural sources [12]. To study these complex sample matrices, screening assays based on liquid chromatography (LC), mass spectrometry (MS), and capillary electrophoresis (CE) were developed [13–15]. In most of these methods, an in-line enzyme bioreactor prepared by immobilizing the enzyme onto a solid support that was retained inside the LC or CE column was used. A test solution containing enzyme substrate was injected and incubated in the enzyme bioreactor for certain time. The product resulting from the enzymatic reaction was then separated from other coexisting components, including unreacted substrate, and was quantified. If an enzyme inhibitor was present in the test solution, the amount of product was decreased. Obviously, having an effective and durable enzyme bioreactor is essential for the success of such a screening method.

To prepare enzyme bioreactors, immobilization techniques were employed, including physical adsorption, ionic binding, covalent binding, and sol-gel entrapment [16]. Wainer and coworkers described the immobilization of enzymes on silica particles that were then packed into LC columns for studying enzyme activity and screening of inhibitors [17]. Enzyme bioreactors prepared by entrapping enzymes in sol-gel-derived monolithic capillary columns were reported for protein analysis by CE [18] and for inhibitor screening by MS [19]. Using glutaraldehyde as a coupling reagent, enzymes were covalently immobilized onto amine-functionalized magnetic nanoparticles to prepare an α-glucosidase bioreactor used for inhibitor screening by CE [20]. In addition,

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[1] *Abbreviations used:* HTS, high-throughput screening; UV, ultraviolet; LC, liquid chromatography; MS, mass spectrometry; CE, capillary electrophoresis; μHPLC–MS/MS, micro-high-performance liquid chromatography–tandem mass spectrometry; GNP, gold nanoparticle; GLDH, L-glutamic dehydrogenase; β-NAD^+, nicotinamide adenine dinucleotide; β-NADH, β-nicotinamide adenine dinucleotide (reduced disodium salt); PEI, poly(ethyleneimine); S/N, signal/noise ratio; RSD, relative standard deviation.

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Zhang and coworkers described a procedure to covalently immobilize trypsin onto a monolithic stationary phase that was activated by glutaraldehyde [21]. The trypsin bioreactor was used in microhigh-performance liquid chromatography–tandem mass spectrometry (μHPLC–MS/MS) proteomic analysis with a digestion speed approximately 6600 times faster than that of digestion in free solutions. Compared with all other immobilization techniques, ionic binding is certainly the most convenient and fastest technique to immobilize enzymes. In an immobilization procedure via ionic binding, the solid support is first coated with a polyelectrolyte to obtain a suitably charged carrier surface (either positively or negatively depending on the predominant charge on the enzyme). The solid support with a charged surface is then soaked with an enzyme solution. Due to the electrostatic interactions, enzyme molecules bind to the surface of the solid support. The coating-and-binding process can be repeated several times, forming a layer-by-layer assembly of enzyme molecules on the carrier surface. Kang and coworkers described a bioreactor of angiotensin-converting enzyme using hexadimethrine bromide as the polyelectrolyte [22] and a layer-by-layer assembly bioreactor of acetylcholinesterase using poly(diallyldimethylammonium chloride) as the polyelectrolyte inside CE columns that were used for enzyme inhibitor screening [23].

Here we describe a new strategy for preparing CE in-column enzyme bioreactors based on the ionic binding technique. The innovative aspect of the proposed procedure is that the enzyme is first immobilized on gold nanoparticles (GNPs) and the functionalized GNPs (instead of enzyme molecules) are then assembled onto the polyelectrolyte-modified carrier surface, forming a GNP-mediated enzyme bioreactor. The rationale for this experimental design is that enzyme loading in the bioreactor mediated by GNPs is expected to be much greater than that prepared from a free enzyme solution because enzyme molecules can be enriched on GNPs with a vast surface-to-mass ratio. In addition, a much better stability of the bioreactor can be expected given that enzyme–GNP conjugates are much more stable than enzyme–polyelectrolyte conjugates. It is well documented that thiol groups (–SH) present in protein molecules bind to GNPs strongly, contributing to the high stability of protein–GNP conjugates [24]. Fig. 1 illustrates an enzyme bioreactor prepared by the proposed approach. In this work, L-glutamic dehydrogenase (GLDH) was selected as the test enzyme. The prepared CE in-column GLDH bioreactor was evaluated in terms of enzyme activity, bioreactor stability, and its usefulness for screening of GLDH inhibitors present in medicinal plants by CE.

Materials and methods

Chemicals and solutions

GLDH, nicotinamide adenine dinucleotide (β-NAD^+), β-nicotinamide adenine dinucleotide (reduced disodium salt) (β-NADH), poly(ethyleneimine) (PEI, MW = 750,000, 50% [w/v] solution), α-ketoglutaric acid, and perphenazine were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA). All medicinal plants tested were collected from local markets. The GLDH solution was prepared by dissolving 0.0020 g of GLDH in 200 μl of 30% glycerol solution and was kept at −20 °C before use. The PEI solution was freshly prepared by diluting the 50% (w/v) PEI solution with water to obtain a concentration of 4% (w/v) PEI.

CE system

All CE experiments were performed using an HP^{3D} CE system (Hewlett Packard, Waldbronn, Germany). UV detection was at a fixed wavelength of 254 nm. Data were processed with an HP ChemStation.

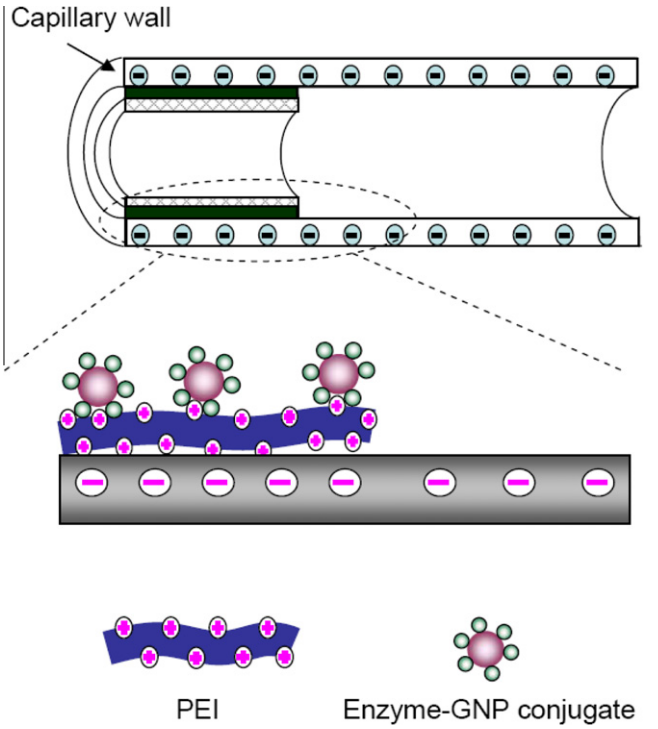

Fig. 1. Schematic of a GNP-mediated enzyme bioreactor prepared in a CE column.

Preparation of GLDH-functionalized GNPs

GNPs (38 nm diameter) were prepared as described previously [25]. Briefly, 100 ml of a 0.01% (w/v) $HAuCl_4$ solution was heated to boiling, and then 1.0 ml of a 1% trisodium citrate solution was added with stirring. The solution was refluxed for 30 min. After cooling to room temperature, the solution was filtered through a 0.45-μm nylon membrane. The pH of the GNP solution was adjusted to 9.0 with K_2CO_3 and kept at 4 °C. The size and monodispersity of GNPs prepared were determined by using a JEOL 100CX transmission electron microscope. To prepare GLDH-functionalized GNPs, 95 μl of the GNP solution was mixed with 5 μl of the above-prepared 10-mg/ml GLDH solution. The mixture was vortexed for 1 min and then let to stand alone at 4 °C for 1 h.

Preparation of a GLDH bioreactor in a CE column

A piece of 50 μm i.d. × 38 cm (29.5 cm to detection window) fused-silica capillary was used. The capillary was treated with a 1 M NaOH solution for 1 h and then flushed with water. An approximately 0.5-cm section of the capillary at the inlet end was filled with the PEI solution as prepared above. After 10 min, the PEI solution was washed out with water. The GLDH-functionalized GNP solution was introduced and filled the PEI-modified section of the capillary. Negatively charged GLDH–GNP conjugates bound to the positively charged PEI coating on a capillary wall. After 2 h, the capillary was flushed with water and ready for use in CE experiments.

Preparation of the extracts of medicinal plants

Air-dried medicinal plants were ground to fine powders. Isopropanol–H_2O (1:1) solution was added to the powder in an Erlenmeyer flask (40 ml isopropanol solution/10 g powder). The mixture was sonicated for 2.5 h at a frequency of 100 kHz and 60 °C by using an ultrasonic cleaning bath. The mixture was

filtered. The filtrate collected was evaporated to nearly complete dryness. The residue was dried in a vacuum dryer at 30 °C and 0.07 MPa, obtaining the extract. Solutions of extracts were prepared in 50% isopropanol at 6 mg /ml. The solution was appropriately diluted with the CE running buffer before assay.

Screening of GLDH inhibitors by CE

The screening assay was based on the following enzymatic reaction:

$$\text{GLDH } \alpha - \text{ketoglutaric acid} + CH_3CO_2NH_4 + \beta\text{-NADH} \rightarrow \text{glutamic acid} + \beta\text{-NAD}^+ + CH_3CO_2H + H_2O$$

For enzyme activity studies, a substrate solution was injected into the CE capillary with an enzyme bioreactor at the inlet end by pressure at 40 mbar for 5 s. A voltage of −28 kV was applied to separate β-NAD^+ from β-NADH and potentially any other existing compounds in the sample solution. After the separation, β-NAD^+ was quantified by UV absorbance at 254 nm. Enzyme activity was assessed based on the amount of β-NAD^+ produced from the enzymatic reaction.

For inhibition studies, the CE running buffer containing a known inhibitor (or extract of a medicinal plant) was injected into the capillary and left in place for 1 min. An inhibitor (or extract)-containing substrate solution was then injected. The CE separation was started, and β-NAD^+ was quantified as described above. The inhibition percentage was calculated according to the peak area of β-NAD^+ compared with the reference electropherogram obtained in the absence of the inhibitor (or extract).

Results and discussion

CE quantification of β-NAD^+

β-NAD^+ is a coenzyme found in all living cells. β-NAD^+ and its reduced form, β-NADH, are involved as a reactant/product pair in many enzymatic reactions. For example, in the GLDH-catalyzed reaction of α-ketoglutaric acid selected in this work for screening of GLDH inhibitors, β-NADH is converted to β-NAD^+. Therefore, quantification of β-NAD^+ in the reaction mixture serves well the purpose of monitoring these enzymatic reactions if β-NAD^+ does not occur in the sample matrix (i.e., the herbal extracts in this work). CE was proven to be a very effective technique to separate these two compounds. Thus, a relatively short CE column (29.5 cm effective length) was used to achieve a fast quantification. Because phosphate ions affect GLDH activity and borate ions do not [26], a borate buffer solution (25 mM at pH 9.0) was chosen as the CE running buffer. Under the experimental conditions selected, β-NAD^+ and β-NADH were separated within 3 min (Fig. 2). For quantification, seven-point calibration curves were prepared by analyzing β-NAD^+ standard solutions at varying concentrations from 0.0100 to 0.400 mM. β-NAD^+ was quantified using the CE peak area. Linear regression resulted in the following regression equation:

$$y = 136x - 0.108,\ r^2 = 0.9993,$$

where y is the peak area, x is the β-NAD^+ concentration (in mM), and r^2 is the correlation coefficient. The detection limit was calculated to be 0.002 mM for β-NAD^+ (signal/noise ratio [S/N] = 3). To determine the assay reproducibility, a 0.050-mM β-NAD^+ standard solution was analyzed 10 times. The assay reproducibility was found to be 1.4% (relative standard deviation [RSD], n = 10). The reproducibility of β-NAD^+ migration time (RSD, n = 10) was 0.65%.

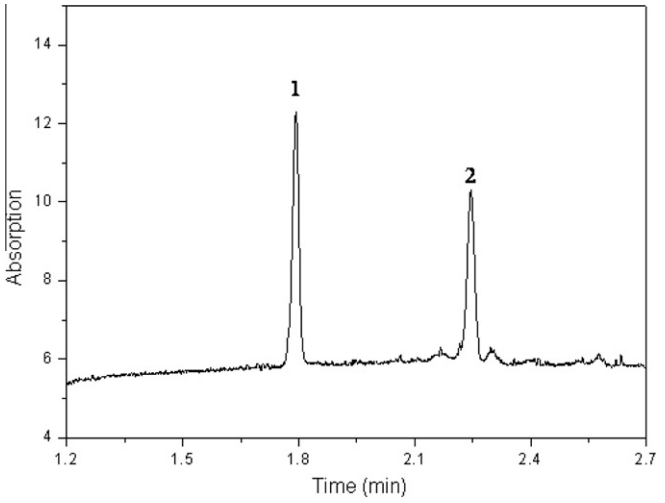

Fig. 2. CE separation of β-NAD^+ and β-NADH. Experimental conditions: CE column, 50 mm i.d. × 29.5 cm effective length fused-silica capillary; injection, 40 mbar for 5 s; running buffer, 25 mM borate buffer (pH 9.0); voltage applied, −28 kV; column temperature, 25 °C; and UV detection at 254 nm. Peak identifications: (1) β-NAD^+ and (2) β-NADH (0.10 mM each).

Performance of in-line GLDH bioreactor

The GLDH bioreactor was first evaluated in terms of GLDH activity of the bioreactor. In these studies, a substrate solution containing 16 mM α-ketoglutaric acid, 0.050 mM β-NADH, and 80 mM ammonium acetate was injected into the CE capillary with an in-line GLDH bioreactor at the inlet end, and the solution was left in the bioreactor for some time (0–3 min) before a CE voltage was applied to separate and quantify β-NAD^+ produced from the enzymatic reaction. The results indicated that the enzymatic reaction was more than 75% complete with zero incubation time (i.e., the CE separation was started immediately after injecting the substrate solution). Fig. 3 shows the relationship between the β-NAD^+ amount produced and the incubation time. Within 1.0 min, the enzymatic reaction was more than 97% complete. The reaction velocity was unexpectedly high considering that the enzyme quantity loaded in the bioreactor was extremely small. The high reaction velocity was likely due to the vast GNP surface area for

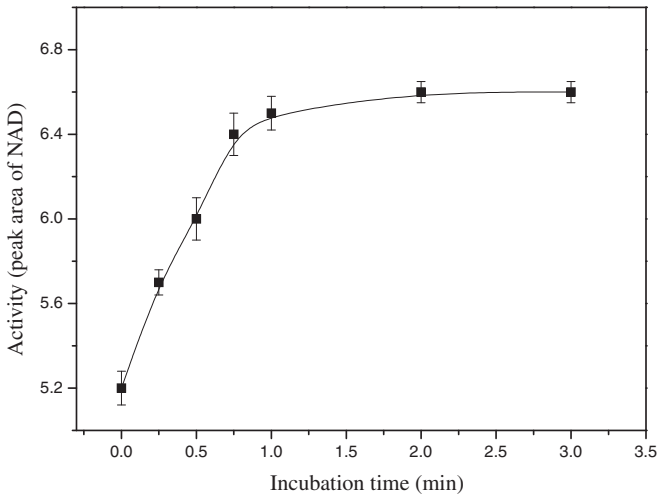

Fig. 3. Enzyme activity tests: incubation time versus amount of β-NAD^+ produced from the enzymatic reaction. The substrate solution injected contained 80 mM ammonium acetate, 16 mM α-ketoglutaric acid, and 0.050 mM β-NADH in CE running buffer. CE experimental conditions were as in Fig. 2

enzyme immobilization and, thus, a high level of contact between the immobilized enzyme and the substrate.

The value of the Michaelis–Menten constant (K_m), an important parameter of an enzymatic reaction, was also determined for the GLDH bioreactor by using the Lineweaver–Burk plotting method:

$$\frac{1}{v} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}, \quad (1)$$

where v and V_{max} are the initial and maximal velocities of the reaction, respectively, and [S] is the substrate concentration. Six β-NADH solutions at different concentrations ranging from 0.020 to 0.100 mM were analyzed. Each of the solutions was analyzed three times. The peak area of β-NAD$^+$ was used to represent the initial reaction rate. A double-reciprocal plot defined by Eq. (1) was constructed. Linear regression analysis of the data produced the following equation: $1/v = 0.373\ (1/[S]) + 24.85$ ($r^2 = 0.9961$). From this equation, K_m was calculated to be 0.015 mM, which was similar to that obtained from the GLDH reaction in free solutions (0.02 mM) [27]. The results suggested that no significant change in the substrate–GLDH binding property was caused by enzyme immobilization.

Stability of the in-line GLDH bioreactor was assessed. A substrate mixture solution was repetitively injected for analysis up to 110 times on the same CE capillary with an in-line GLDH bioreactor. Fig. 4 shows the results of the β-NAD$^+$ peak area versus the number of assays. As can bee seen, the efficacy of the GLDH bioreactor (measured by β-NAD$^+$ peak area) tended to decrease as the number of assays increased. However, the decrease was relatively insignificant (<15% after an extensive use of 110 times). In addition, the bioreactor could be regenerated easily by dipping the inlet end of the capillary into the GLDH-functionalized GNP solution for 10 s and flushing the capillary with water 2 h afterward. For comparison, a GLDH bioreactor was prepared by immobilizing GLDH (instead of GLDH-functionalized GNPs) onto the inner wall of capillary treated with PEI. It was found that the enzymatic activity decreased very quickly. The bioreactor lost approximately 40% of its initial activity after only five assays. The improved stability of the GNP-mediated bioreactor likely resulted from the fact that the GLDH–GNP conjugate was much more stable than the GLDH–PEI conjugate.

The above-described studies showed that the in-line GLDH bioreactor had not only a high enzymatic activity but also a good stability. In addition, the CE quantification allowed accurate, reliable, and quick monitoring of the GLDH reaction. These results combined suggested that the CE-based method was well suited for screening of GLDH inhibitors. To evaluate its applicability for GLDH inhibitor screening, inhibition studies using a known GLDH inhibitor, perphenazine, were performed. In these tests, a perphenazine solution prepared in the CE running buffer was injected and left in place for 1 min. The substrate solution containing perphenazine at 0.125 mM was then injected. It was noted that without the inhibitor in the bioreactor, a significant amount of the substrate was converted to the product before the enzyme was inhibited due to a very fast enzymatic reaction rate. The inhibition percentage was calculated using the following equation:

$$\text{Inhibition } \% = 100 - \left(\frac{x}{\text{blank}} \times 100\right) \quad (2)$$

where x and blank are the peak areas of β-NAD$^+$ measured with and without the inhibitor, respectively. The quality of screening assays can be assessed by using the Z' factor, calculated as follows [28]:

$$Z' = 1 - \frac{3\sigma_s + 3\sigma_c}{|\mu_s - \mu_c|} \quad (3)$$

where μ_s and μ_c are the means of the signal (i.e., β-NAD$^+$ peak area in this work) obtained from the standard assay (in the absence of inhibition) and the control assay (100% inhibition by a known inhibitor), respectively, and σ_s and σ_c are the standard deviations of the data. A Z' factor value between 0.5 and 1 indicates that the quality of the screening data is excellent (i.e., the inhibitor screening assay is accurate and reliable). In this work, perphenazine solutions at 0.06 and 0.12 mM were assayed. The Z' factor value was calculated as 0.95 (n = 10). The result indicated that the accuracy of the current CE-based screening method was excellent for the screening of GLDH inhibitors.

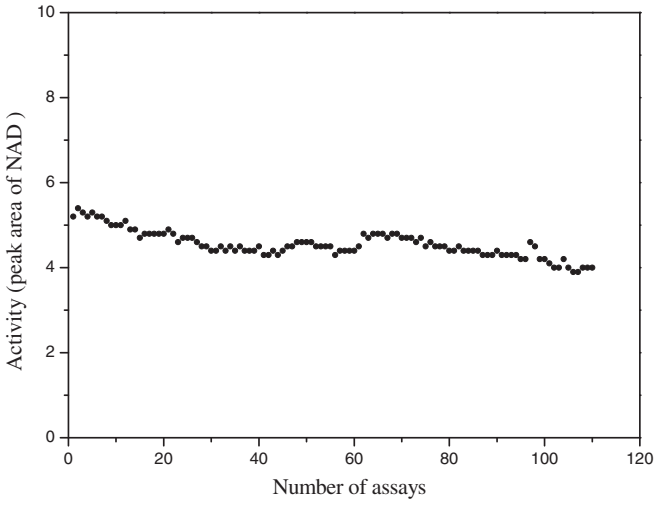

Fig. 4. Stability of the in-line GLDH bioreactor: enzyme efficacy versus number of assays. Experimental conditions were as in Fig. 3.

Screening of GLDH inhibitors in Chinese medicinal plants

Isopropanol extracts were prepared from 25 Chinese medicinal plants. In addition, perphenazine (a known GLDH inhibitor) was also included in the chemical library for the purpose of assay validation (as a positive control). Triplicate assays were made for each isopropanol extract. The averaged peak areas from the three measurements were used to calculate the inhibition percentage by Eq. (2). Fig. 5 shows several electropherograms from the screening assays. Fig. 5A was from a blank solution (i.e., in the absence of inhibition). The injected substrate solution contained α-ketoglutaric acid, ammonium acetate, and β-NADH. Fig. 5B and C were from assaying *Radix scutellariae* and *Radix codonopsis* extracts (both at 0.6 mg/ml), respectively. The injected sample solutions were the CE running buffer containing the extract at 0.6 mg/ml for preincubation and then the substrate solution containing the extract. As can be seen, the peak areas of β-NAD$^+$, the product of the enzymatic reaction, were clearly reduced compared with that in Fig. 5A from a blank solution, clearly indicating GLDH inhibition (53% inhibition from *R. scutellariae* extract and 45% from *R. codonopsis* extract). It is worth noting that several endogenous compounds present in the extract were separated from β-NAD$^+$ and β-NADH, as shown in Fig. 5B. This might eliminate potential interference with the quantification and, therefore, false screening results. As can be seen in Table 1, perphenazine (at 0.125 mM) and three extracts at 0.6 mg/ml were found to be positive for GLDH inhibition. The other extracts did not show any inhibitory effects. They served well as negative controls for the screening assay, whereas perphenazine served as a positive control. The extract of *R. scutellariae* showed a 53% inhibition on GLDH activity. *R. scutellariae* is widely used for the treatment of inflammation, fever, hepatitis, allergic diseases, hypertension, and the like in Chinese traditional medicine [29]. Studies have shown that

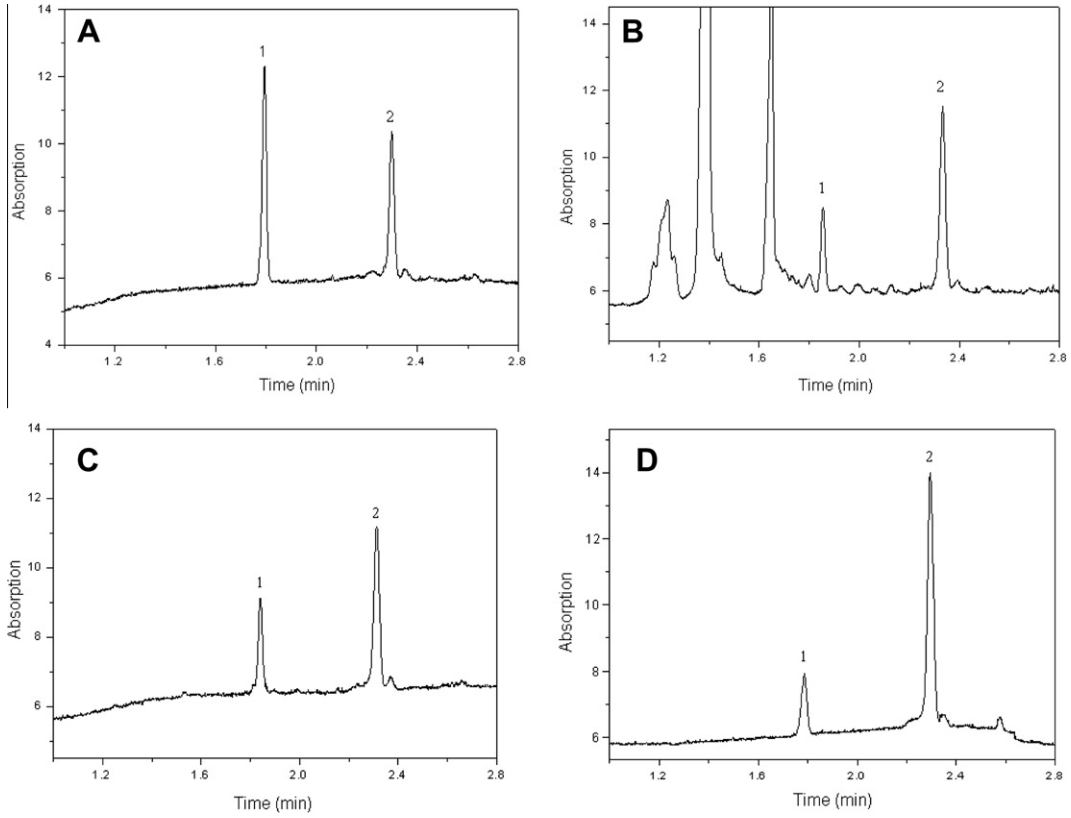

Fig. 5. Electropherograms obtained from the screening assays: (A) blank solution (i.e., the substrate solution); (B) *Radix scutellariae* extract at 0.6 mg/ml; (C) *Radix codonopsis* extract at 0.6 mg/ml; (D) perphenazine (a known GLDH inhibitor) at 0.125 mM. Assay conditions were as in Fig. 3. Peak identifications: (1) β-NAD^+ and (2) β-NADH.

Table 1
Results of GLDH inhibitor screening in medicinal plant extracts.

Medicinal plant	Inhibition %[a]	Medicinal plant	Inhibition %[a]
Perphenazine (a known GLDH inhibitor)	65	*Exocarpium citri rubrum*	0
Radix astagali	0	*Wrinkled gianthyssop*	0
Fructus crataegi	0	*Herba verbenae*	0
Poria	0	*Radix scutellariae*	53
Radix achyranthis bidentatae	0	*Radix scrophulariae*	0
Fructus hordei germinatus	0	*Semen lablab album*	0
Radix paeoniae alba	37	*Pericarpium citri reticulatae*	0
Fructus piperis	0	*Leaf of Henon bamboo*	0
Rhizoma chuanxiong	0	*Fructus amomi rotundus*	0
Rhizoma anemarrhenae	0	*Radix et rhizoma rhei*	0
Radix ophiopogonis	0	*Semen arecae*	0
Rhizoma pinelliae	0	*Radix codonopsis*	45
Rhizoma atractylodis macrocephalae	0	*Bulbus fritillariae cirrhosae*	0

Note. The concentration of extract was 0.6 mg/ml, and the concentration of perphenazine was 0.125 mM.
[a] Means of triplicate assays.

R. scutellariae lowers blood pressure and has sedative effects on the central nervous system [30,31].

Conclusion

GLDH could be easily immobilized on 38-nm-diameter GNPs. The functionalized GNPs were assembled on the inner wall at the inlet end of a CE capillary modified by PEI, producing an in-line GLDH bioreactor. The GNP-mediated GLDH bioreactor showed a high enzymatic activity and a very good stability. The value of the Michaelis–Menten constant (K_m) was 0.015 mM, which was very similar to that obtained from the GLDH reaction in free solutions. The result suggested that no significant changes in the substrate–GLDH binding property were caused by enzyme immobilization. Use of the highly effective and durable in-line GLDH bioreactor in combination with the proposed CE quantification of β-NAD^+ allowed accurate and reliable GLDH inhibitor screening. The proposed screening method was applied to analyzing extracts of 25 medicinal plants. The inhibition percentages were found to be 53% for *R. scutellariae*, 45% for *R. codonopsis*, 37% for *Radix paeoniae alba*, and 0% for the other 22 extracts tested. The screening method was proved to be accurate, easy to carry out, and well suited for assaying complex samples such as medicinal plant extracts.

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