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# Online screening of acetylcholinesterase inhibitors in natural products using monolith-based immobilized capillary enzyme reactors combined with liquid chromatography-mass spectrometry

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## ABSTRACT

In order to develop a direct and reliable method for discovering lead compounds from traditional Chinese medicines (TCMs), a comparative online ligand fishing platform was developed using immobilized capillary enzyme reactors (ICERs) in combination with liquid chromatography-mass spectrometry (LC-MS). Methacrylate-based monolithic capillaries (400 μm I.D. × 10 cm) containing epoxy reactive groups were used as support to immobilize the target enzyme acetylcholinesterase (AChE). The activity and kinetic parameters of the AChE-ICER were investigated using micro-LC-UV. Subsequently, ligand fishing and identification from mixtures was carried out using the complete AChE-ICER-LC-MS platform. For efficient distinction of true actives from false positives, highly automated comparative analyses were run alternately using AChE-ICERs and negative control-ICERs, both online installed in the system. After washing unbound compounds to the waste, bound ligands were eluted from the AChE-ICER to a trapping loop using a denaturing solution. The trapped ligands were further separated and identified using LC-MS. Non-specific binding to the monolith support or non-functional sites of the immobilized enzyme was investigated by exposing analytes to the negative control-ICER. The specificity of the proposed approach was verified by analyzing a known AChE inhibitor in the presence of an inactive compound. The platform was applied to screen for AChE inhibitors in extracts of *Corydalis yanhusuo*. Eight compounds (columbamine, jatrorrhizine, coptisine, palmatine, berberine, dehydrocorydaline, tetrahydropalmatine and corydaline) with AChE binding affinity were detected and identified, and their AChE inhibitory activities were further verified by an *in vitro* enzymatic inhibition assay. Experimental results show that the proposed comparative online ligand fishing platform is suitable for rapid screening and mass-selective detection of AChE inhibitors in complex mixtures.

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## 1. Introduction

Traditional Chinese medicines (TCMs) are attracting increasing attention all over the world, due to their long historical clinical practice and appealing therapeutic efficacy. Moreover, TCMs possess high chemical scaffold diversity and can be considered as a huge and invaluable source of bioactive compounds for discovering promising new drugs [1,2]. However, because of the chemical

complexity of TCMs, it is neither easy to identify bioactive constituents nor to elucidate their pharmacological mechanism. The conventional bioassay-guided fractionation approach has been a mainstream method for discovering bioactive compounds from natural products. Unfortunately, the isolation procedures central to this approach are usually labor-intensive, time-consuming and costly, and in many cases lead to the loss of bioactive compounds due to dilution and decomposition as well as sticking to vials, tubes, etc. [3]. Therefore, it is desirable to establish reliable and rapid methods for screening and identifying bioactive constituents from TCMs directly.

Taking advantage of good selectivity and high throughput, affinity-based approaches coupled to advanced chemical detectors have been frequently used to screen bioactive compounds in

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TCMs [4–7]. Ligand fishing is a well-developed affinity-based technique in which selective binding of ligands to target enzymes or receptors allows separation from unbound components of TCMs. The bound ligands are subsequently dissociated and identified using liquid chromatography-mass spectrometry (LC–MS). Up to now, ligand fishing experiments have been carried out in different formats, including ultrafiltration [8], equilibrium dialysis [9], nanotubes [10], magnetic beads [11,12], zeolite [13] and hollow fibers [14]. These methods are mostly applied in an offline mode, which often is tedious and suffers from time-consuming analytical steps involving incubation, separation, dissociation, and analysis. Online ligand fishing may be more attractive, since the incubation, ligand-enzyme/receptor complex isolation, dissociation and HPLC–MS analysis can be carried out in a continuous, automated fashion, which can greatly enhance the screening efficiency [15].

Affinity based solid-phase extraction columns, which use enzyme-functionalized media for capturing potential ligands, have been employed for online ligand fishing. Jonker et al. used dynamic protein-affinity chromatography solid-phase extraction (DPAC-SPE) combined with LC–MS for screening and identifying estrogen receptor alpha (ER $\alpha$ ) ligands in complex mixtures. However, this DPAC-SPE method can only be used for fishing of His-tagged proteins [16]. Recently, Peng et al. established online coupling of an affinity SPE column with LC–MS/MS for fishing xanthine oxidase (XO) inhibitors which allowed rapid isolation and identification of inhibitors from complex mixtures [17]. However, the efficient packing of affinity SPE columns, particularly in micro- and capillary format, can be difficult. Polymeric monoliths have shown to be a highly useful alternative support material to immobilize proteins for e.g. proteomics studies [18], ligand-protein binding studies, and ligand affinity ranking studies [19–21]. So far, the use of monolith-based immobilized capillary enzyme reactors (ICERs) for online ligand fishing, particularly in relation to TCM profiling, has not been reported.

When applying ligand fishing methods, due attention should be paid to the prevention of false positives caused by non-specific binding of compounds to the support material and/or non-functional sites of the enzyme [11,22]. Recently, Chen et al. developed an online comparative cell membrane chromatography (CMC) method by simultaneously using CMC columns packed with normal and pathological tissue-derived silica. This approach effectively increased the specificity of the screening results through visualized comparison of the chromatographic affinity behaviors between normal and pathological CMC columns [23].

Acetylcholinesterase (AChE) can terminate nerve impulse by hydrolyzing active neurotransmitter acetylcholine (ACh) in central nervous system (CNS) [24]. The inhibition of AChE from breaking down acetylcholine (ACh) is one of the most important therapeutic strategies in Alzheimer's disease treatment. Furthermore, AChE inhibitors can be used as insecticides to kill insects [25,26]. It is of importance to find new inhibitors that could modulate AChE activity. Some AChE-based immobilized enzyme reactors (AChE-IMERs) have already been developed for screening AChE inhibitors from pure compound library or assessing the overall inhibitory activity of natural products [27–30]. For example, Bartolini et al. developed a human recombinant AChE micro-immobilized enzyme reactor (hrAChE-IMER) by immobilizing hrAChE on monolithic disk (12mm  $\times$  3mm i.d.) [31]. The prepared hrAChE-IMER allowed to screen potential hrAChE inhibitors rapidly from pure compound library, but it was not used as SPE column to directly fish ligands in natural products.

In this study, AChE-ICERs and control-ICERs were prepared through immobilizing AChE onto the surface of a poly (glycidyl methacrylate-co-ethylene dimethacrylate) (poly (GMA-co-EDMA)) monolithic support through a ring opening reaction between epoxy groups and amine groups. The resulting AChE-ICER and control-

ICER were installed in parallel as SPE columns to establish a comparative online ligand fishing platform for rapid separation and identification of AChE ligands in TCMs (as shown in Fig. 1). With this system, ligands are first captured on the AChE-ICER, while inactive compounds are flushed to waste by washing buffer. For identification, the bound ligands are desorbed and eluted to LC–MS through valve switching. Parallel comparison is conducted by performing two subsequent analytical runs on the different SPE columns to eliminate false results caused by non-specific binding. The applicability of this comparative online ligand fishing platform was tested by screening AChE inhibitors from extracts of *Corydalis yanhusuo*. The activity of the found ligands was verified by an AChE inhibitory assay.

## 2. Experimental

### 2.1. Chemicals and materials

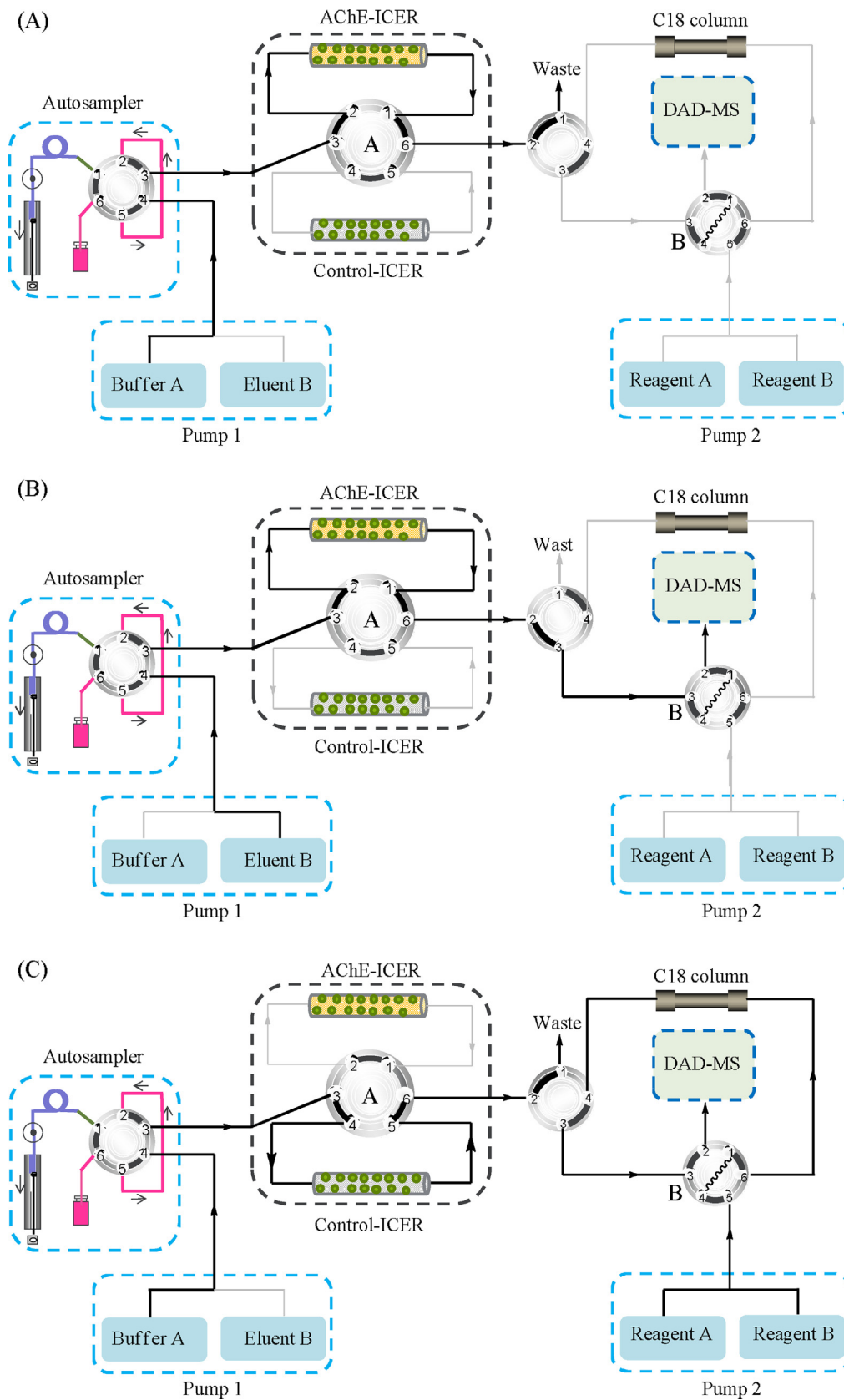
Acetylcholinesterase from *Electrophorus electricus* (eelAChE) type VI-S, acetylthiocholine iodide (ATCh) and 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent) were purchased from Sigma-Aldrich (Shanghai, China). 3-(trimethoxysilyl)propyl methacrylate ( $\gamma$ -MAPS), 2,2'-azobisisobutyronitrile (AIBN), glycidyl methacrylate (GMA), ethylene dimethacrylate (EDMA), galantamine, 1,4-butanediol, 1-propanol and ammonium acetate were all purchased from Aladdin Chemicals (Shanghai, China). Acebutolol hydrochloride was obtained from the Guangdong Institute for Food and Drug Control (Guangzhou, China). Coptisine, berberine, jatrorrhizine hydrochloride, palmatine hydrochloride, cymbamine, dehydrocorydaline, tetrahydropalmatine and corydaline were purchased from Shanghai Yuanye Bio-Technology Co. Ltd. HPLC-grade methanol (MeOH) and acetonitrile (ACN) were obtained from Merck (Shanghai, China). The fused-silica capillaries with an inner diameter of 400  $\mu$ m (800  $\mu$ m O.D.) were purchased from Polymicro Technologies Ltd. (Phoenix, AZ, USA).

### 2.2. Instrumentation

Enzyme immobilization was carried out by using a precise peristaltic pump (Baoding Longer Pump Company, Hebei, China) with a 2 mL syringe. A Jinghong DK-S22 water bath (Shanghai, China) was used for the thermally initiated co-polymerization. All scanning electron microscopy (SEM) experiments were performed on a Leo 1530 VP Field Emission Scanning Electron Microscope equipped with Oxford INCA 400 energy dispersive X-ray microanalysis (Oberkochen, Germany) at an acceleration voltage of 1 kV. The *in vitro* AChE inhibitory assays were conducted on a microplate reader (Biotek Instrument, USA).

The immobilized enzyme catalytic activity assays were performed by installing the ICERs on a self-assembled micro-LC system, which is composed of a DiNa-S nano pump (Tokyo, Japan), a Dionex Spark 920 LC Packing Famos Autosampler (Emmen, Netherlands) with 1  $\mu$ L sample loop and an Applied Biosystems 785A UV-vis detector (USA). LC–MS experiments were carried out on an Agilent 1260 Infinity Series HPLC analytical system coupled to Agilent 6540 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The 1260 infinity HPLC analytical system consisted of a quaternary pump, a degasser, an autosampler with 5  $\mu$ L sample loop, a column oven, a six-port dual-position valve and a diode array detector.

The online platform is an integration of some modules of the 1260 infinity HPLC analytical system (Agilent Technologies, USA) and a PU-1580 Jasco binary pump (Kyoto, Japan). Two additional Valco two position switching valves with a four-port and a six-port (Houston, TX, USA), respectively, were used for con-



**Fig. 1.** Schematic diagram of the online ligand fishing and identification platform. (A) Ligands are trapped from the sample solution and separated from inactive compounds by AChE-ICER. (B) The ligands bound on the ICERs are desorbed and led to the loop on six-port valve B. (C) Ligands are directed to the LC-MS system and analyzed; simultaneously, sample solution is led over the control-ICER, which subsequently is desorbed and eluted compounds are analyzed by LC-MS.

necting different modules. Data acquisition and handling were performed using Unimicro Trisep™ Workstation 2003 (Shanghai, China) and MassHunter software (Agilent Technologies, USA). All chromatograms were converted to a text file and redrawn using Microcal Origin 8.5.

### 2.3. Preparation of poly (GMA-co-EDMA) monolithic support

The inner surface of the fused-silica capillaries (400  $\mu\text{m}$  I.D.  $\times$  800  $\mu\text{m}$  O.D.) was first pretreated with  $\gamma$ -MAPS to enable covalent attachment of the bulk polymer to the capillary wall. The polymerization mixture composed of functional monomer (21% GMA, 14% EDMA), AIBN (an initiator, 1% with respect to the monomers) and porogens (4.5%  $\text{H}_2\text{O}$ , 15.8% 1,4-butanediol and 44.7% 1-propanol) (all percentages are in w/w) was sonicated for 5 min to obtain a homogenous solution, and then the solution was introduced into the pretreated capillaries. With both ends of the capillaries sealed by rubber, the thermally initiated polymerization was carried out in a water bath at 65 °C for 12 h. Finally, the non-reacted monomers and porogens were flushed out with MeOH.

### 2.4. Preparation of AChE-ICERs

Before the immobilization, the poly (GMA-co-EDMA) monolith was equilibrated for 10 min with immobilization buffer (15 mM ammonium acetate (pH 8.0)). Afterwards, a solution of 0.5 mg/mL AChE in immobilization buffer was pumped through the monolith continuously at a flow rate of 60  $\mu\text{L}/\text{h}$  for 3 h. Finally, the resulting AChE-ICER was washed with water to eliminate non-specifically adsorbed enzyme, and stored at 4 °C when not in use. In addition, a negative control-ICER column was prepared by exposing an AChE-ICER column for 3 h to 80 °C in a water bath in order to denature (i.e. deactivate) the enzyme.

### 2.5. Enzyme activity assays

The activity of the AChE-ICER was tested using a micro-LC-UV system. 10 mM ammonium acetate (pH 7.4) containing 0.126 mM Ellman's reagent was used as mobile phase and the flow rate was 5  $\mu\text{L}/\text{min}$ . The catalytic activity of the immobilized AChE and free AChE were determined by measuring the formation of yellow anion (YA) at 412 nm [32]. The YA was produced by the reaction between Ellman's reagent and the enzymatic product thiocholine. The activity of free AChE was set to 100%, and the relative enzyme activity was determined by comparing the activities between the immobilized AChE with free AChE.

The kinetic parameters can be calculated by using Lineweaver-Burk plots  $1/V_0 = (K_m/V_{\text{max}}) \cdot (1/[S]) + 1/V_{\text{max}}$ , where  $V_0$  and  $V_{\text{max}}$  are the initial and maximal reaction velocities,  $[S]$  is the concentration of substrate, and  $K_m$  is the Michaelis-Menten constant [33]. The  $K_m$  for the immobilized AChE was determined by injecting 1  $\mu\text{L}$  substrate ATCh in aqueous solution at the concentration range of 0.005–5 mM. In order to determine the inhibitory potency ( $\text{IC}_{50}$ ), 0.1 mM of ATCh with increasing concentration of inhibitor (0.2–500  $\mu\text{M}$ ) was injected into both the AChE-ICER and the negative control-ICER, respectively. The following formula was employed to calculate the  $\text{IC}_{50}$  values: Inhibition (%) =  $100 - (P_i - P_c)/(P_0 - P_b) \times 100$ , where  $P_0$  and  $P_i$  are the peak areas of YA determined in the absence or presence of inhibitors with the AChE-ICER,  $P_b$  and  $P_c$  are the peak areas of YA determined in the absence or presence of inhibitors with the negative control-ICER, respectively. All samples were analyzed three times. Origin Pro 8.5 software was used to calculate  $K_m$  according to Lineweaver-Burk plots and fit the inhibition curve.

### 2.6. Setup of comparative online ligand fishing and identification platform

The comparative online ligand fishing platform is composed of commercially available modules (as shown in Fig. 1). The AChE-ICER and the negative control-ICER were applied as 'trapping' columns. In Fig. 1, the six-port valve used to connect the ICERs is presented as valve A, and the other six-port valve, in the LC-MS system, is presented as valve B. Procedures for online ligand fishing and subsequent identification of the active compounds are as follows:

Step 1 (Fig. 1A): All valves including six-port valve A, six-port valve B, and the four-port valve are in position 1, and buffer A (10 mM ammonium acetate (pH 7.4)) is pumped by pump 1. An aliquot of 3  $\mu\text{L}$  of the sample solution is loaded on the AChE-ICER, and non-bound fraction is washed away using 10 mM ammonium acetate (pH 7.4) at a flow rate of 5  $\mu\text{L}/\text{min}$  for 40 min.

Step 2 (Fig. 1B): The four-port valve is switched to position 2, and buffer A is replaced by eluent B (MeOH). The bound ligands on the AChE-ICER are desorbed with MeOH at a flow rate of 20  $\mu\text{L}/\text{min}$  for 13 min and flushed into a 290- $\mu\text{L}$  sample loop at valve B.

Step 3 (Fig. 1C): Six-port valve B in the LC-MS system is switched to position 2, and the compounds in the sample loop are introduced to the C18 column for separation and diode array-MS detection.

The same procedure is repeated for the negative control-ICER, with the six-port valve A switched to position 2.

### 2.7. LC system for validation of the comparative online ligand fishing platform

The online ligand fishing procedures were performed as described above. Chromatographic separation of a mixture of galantamine (AChE inhibitor) and acebutolol (non-AChE inhibitor) was carried out on a SunFire™ C18 column (250 mm  $\times$  4.6 mm, 5  $\mu\text{m}$ , Waters) with isocratic elution of 55% solvent A (0.2 M potassium dihydrogen phosphate (pH 6.8), adjusted with 1 M NaOH) and 45% solvent B (MeOH). The flow rate, injection volume and column temperature were 1.0 mL/min, 3  $\mu\text{L}$  and 25 °C, respectively. The online eluted compounds were analyzed using the subsequent online LC with UV absorbance detection at 290 nm.

### 2.8. Application to *Corydalis yanhusuo* extract

#### 2.8.1. Preparation of *Corydalis yanhusuo* extract

Dried tubers of *Corydalis yanhusuo* were purchased from a local drugstore. The sample preparation procedure was as follows. Firstly, the dried tubers of *Corydalis yanhusuo* were ground into powder. About 6 g of the powder was weighed and extracted 3 times by reflux with 240 mL of 70% ethanol in a round bottom flask for 1.5 h each time. Next, the extracts were pooled and evaporated to 20 mL by rotary vaporization at 55 °C under reduced pressure. The pH of the concentrated extract was adjusted to 2 with HCl. After filtration, the pH of the filtrate was raised to 12 with NaOH and then extracted three times with 100 mL ethyl acetate. Then, the ethyl acetate fraction was collected and evaporated to dryness by rotary vaporization at 55 °C under reduced pressure. Finally, the residue was dissolved in water with 10% ethanol to a concentration of 3 mg/mL, and then filtered through a 0.45  $\mu\text{m}$  filter membrane and stored at 4 °C for further experiments.

#### 2.8.2. Ligand fishing from *Corydalis yanhusuo* extract

An aliquot of 3  $\mu\text{L}$  of the *Corydalis yanhusuo* extract dissolved in water with 10% ethanol was injected, and the ligand fishing procedures of the complete online ligand fishing platform were carried out as shown in Fig. 1. For LC-MS an XTerra MS C18 column (250 mm  $\times$  4.6 mm, 5  $\mu\text{m}$ , Waters) was employed. Gradient

elution solvent A (0.2% acetic acid in water, adjusted with ammonium hydroxide to pH 5.0) and B (ACN) was applied as follows: 0–15 min, 20% B; 15–35 min, 20–80% B; 35–37 min, 80%–20% B. The flow rate and column temperature were 1.0 mL/min and 25 °C, respectively. The diode array detector was monitored at 280 nm.

The mass spectrometer was run in positive ion mode and the ESI source conditions were as follows: capillary voltage, 4000 V; nozzle voltage, 1000 V; nebulizer pressure, 40 psi; temperature, 350 °C; drying gas flow, 10 L/min; sheath gas flow, 12 L/min; fragmentor voltage, 100 V. The mass spectra were recorded for the range of  $m/z$  100–600.

### 3. Results and discussion

#### 3.1. Preparation of the poly (GMA-co-EDMA) monolith

Poly (GMA-co-EDMA) monolith has been commonly employed as the support of choice for immobilizing biological agents. This is not only because of advantages related to organic monoliths, such as simple preparation and high stability under diverse pH conditions (pH 2–12), but also due to presence of highly reactive epoxy groups [34,35]. Various binding agents, such as enzymes and receptors, can be easily introduced to this type of monolithic surface via a ring opening reaction with epoxy groups [36]. Therefore, the poly (GMA-co-EDMA) monoliths were selected in the current study for immobilizing AChE. In order to increase the amount of immobilized enzyme for ligand fishing, wide internal diameter fused silica capillaries (400  $\mu\text{m}$  I.D.  $\times$  800  $\mu\text{m}$  O.D.) were used instead of more common 100  $\mu\text{m}$  I.D. capillaries.

With reference to previous reported methods for preparing poly (GMA-co-EDMA) monolith, a polymerization mixture composed of the functional monomers (GMA and EDMA), ternary porogens (1,4-butanediol, 1-propanol and  $\text{H}_2\text{O}$ ) and initiator AIBN was employed [37]. Considering that the optimal polymerization conditions within 100 and 400  $\mu\text{m}$  I.D. fused silica capillaries may be different due to the possible radical temperature gradient across the tube diameter during the exothermic *in situ* polymerization process, the composition of the polymerization mixture was systematically re-optimized. Finally, a polymerization mixture containing 35% wt. monomers (GMA:EDMA, 60:40, w/w) and 65% wt. porogens (1,4-butanediol:1-propanol: $\text{H}_2\text{O}$ , 24.3:68.8:6.9, w/w) was selected for preparing the poly (GMA-co-EDMA) monolithic columns (400  $\mu\text{m}$  I.D.).

#### 3.2. Optimization of AChE immobilization

In order to successfully immobilize AChE, several parameters including the AChE concentration and immobilization time were systematically optimized. Different concentrations of AChE (0.2, 0.5, 1.0 mg/mL) in 15 mM ammonium acetate (pH 8.0) were pumped through the monolith for a constant time. The relative enzyme activity increased significantly with increasing AChE concentrations from 0.2 to 0.5 mg/mL (Fig. 2A). There was no significant change of the relative enzyme activity when the AChE concentration was further increased to 1.0 mg/mL. Therefore, 0.5 mg/mL AChE was selected for enzyme immobilization. Fig. 2B shows the influence of the immobilization time (1, 3, 6, 12 h) on the relative enzyme activity. A maximum relative enzyme activity was reached at 3 h. Longer immobilization times did not increase the relative enzyme activity, or even led to a decrease at 12 h. This may be ascribed to an increase in steric hindrance caused by binding of excessive enzyme, which can be detrimental to the exposure of the enzyme active sites [38].

The immobilization reproducibility of the optimized AChE-ICERs was evaluated through calculating the RSD values of the

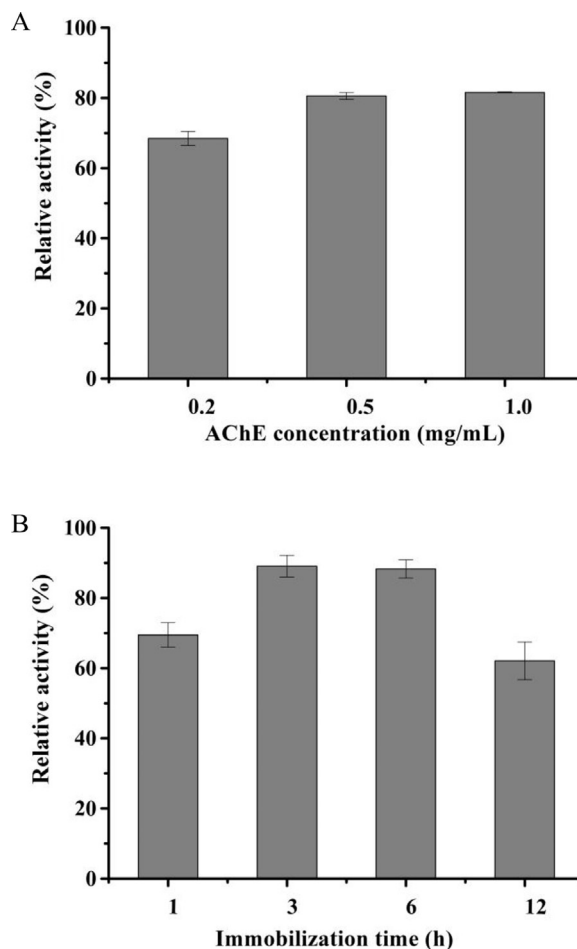


Fig. 2. Effect of the AChE concentration (A) and the immobilization time (B) on relative enzyme activity during the enzyme immobilization procedure.

immobilized enzyme catalytic activity between different batches. The batch-to-batch ( $n=5$ ) RSD value was 4.10%, which demonstrated that the AChE-ICERs have a satisfactory immobilization reproducibility. The stability of the AChE-ICERs was investigated by comparing its catalytic capability with that of free AChE after one weeks' storage. The results showed that the immobilized AChE could maintain more than 70% of its original activity for at least one week, while the free AChE loses its original catalytic activity rapidly to less than 35%.

#### 3.3. Morphologic characterization of monolith

The monolithic microstructure, which may affect on permeability and enzyme immobilization [39,40] was evaluated. Fig. 3A shows the scanning electron micrographs of the optimized poly (GMA-co-EDMA) monolith. The monolithic matrix is tightly anchored to the inner wall of the fused capillary, and a large amount of homogeneous spherical units agglomerated into larger clusters interspersed by large-pore channels, which is characteristic of monolithic structures. After immobilizing with AChE, the surface of clusters seems to have become rougher, while there are no clear changes in the size and uniformity of spherical units (Fig. 3B).

#### 3.4. Kinetic studies

Covalent attachment of enzyme to the surface of monolithic supports may affect its ultimate activity [41]. In order to verify the

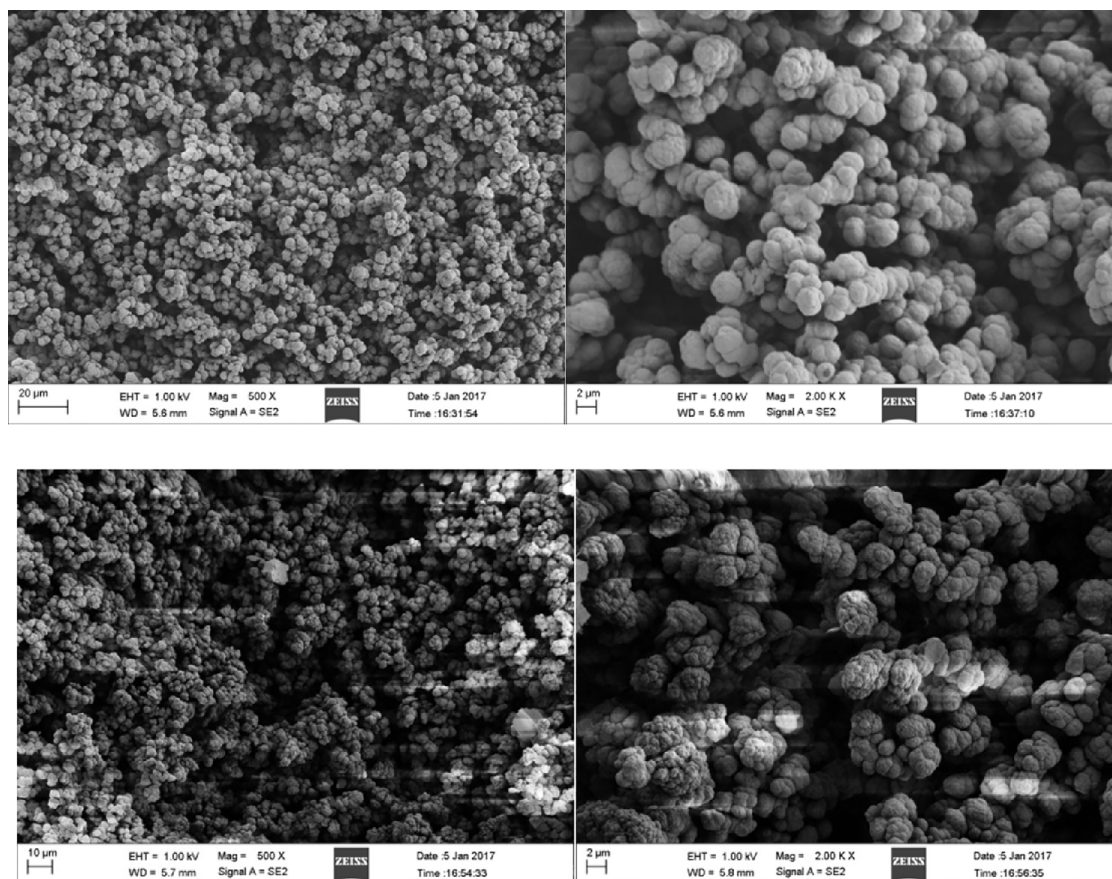


Fig. 3. SEM images of the cross-sections of the poly (GMA-co-EDAM) monolith (A) and the AChE immobilized monolith (B).

catalytic activity of the immobilized AChE, several kinetic parameters  $K_m$ ,  $V_{max}$  and  $IC_{50}$  were determined.

The  $K_m$  and  $V_{max}$  values of the optimized AChE-ICER were determined by varying the substrate ATCh concentration under the described experimental conditions. The Lineweaver-Burk plots of the double reciprocal plots of  $1/[\text{velocity}]$  and  $1/[\text{substrate}]$  was found to be  $y = 9.454 \times 10^{-8}x + 11.607 \times 10^{-8}$  ( $r^2 = 0.9924$ ), while the  $V_{max}$  value (the activity of enzyme reactors) was calculated to be  $8.615 \times 10^6 \pm 0.783 \times 10^6$  mM/min. The  $K_m$  value, which reflects the enzymatic affinities, was determined to be  $0.815 \pm 0.072$  mM (Fig. 4A). This  $K_m$  value was in the range of reported values for other immobilized AChE systems [27,42], showing functional activity of the optimized AChE-ICER for the substrate tested.

Galantamine was selected as test AChE inhibitor for assessing the performance of the AChE-ICER for ligand affinity profiling. Inhibition of enzymatic activity by galantamine was measured in the concentration range 0.2–500  $\mu\text{M}$ . As shown in the resulting dose-response curve (Fig. 4B), the enzymatic activity decreased with increasing concentrations of the inhibitor galantamine, demonstrating that AChE activity was indeed efficiently inhibited by galantamine. The  $IC_{50}$  value was determined to be  $7.17 \pm 0.04$   $\mu\text{M}$ , which is in accordance with reported data [28], indicating that the immobilized AChE has similar enzymatic properties as AChE in solution.

### 3.5. Optimization and evaluation of the comparative online ligand fishing platform

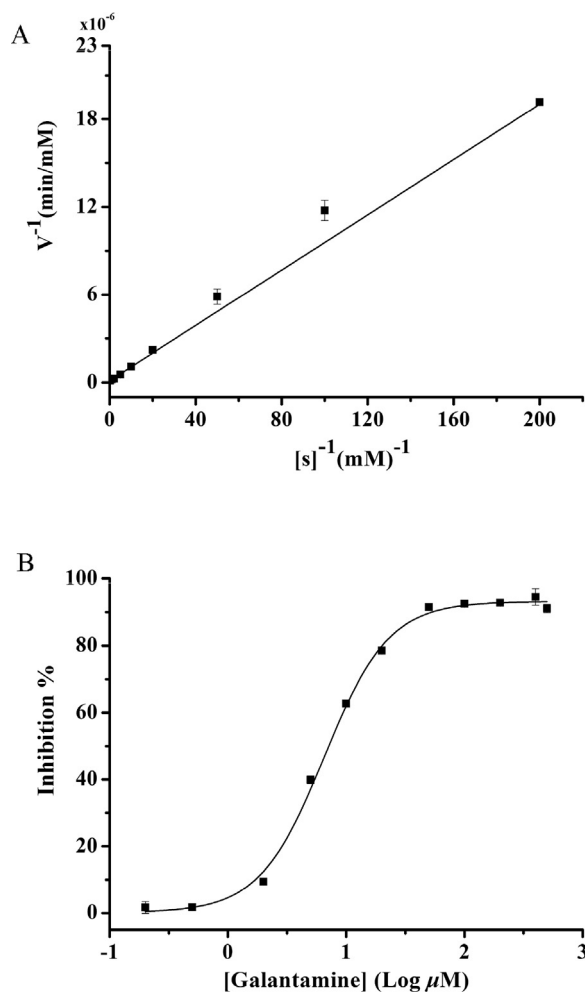
For a successful ligand fishing, it is essential to guarantee sufficient specific interaction time between bioactive compounds and the immobilized enzyme and minimize non-specific binding of

non-binders. Therefore, different parameters important for the AChE-ICER incubation process, including the volume and flow rate of the wash buffer (10 mM ammonium acetate (pH 7.4)) were investigated. Experiments showed that a wash buffer volume of 165  $\mu\text{L}$  was able to efficiently transfer the non-AChE binding compound acebutolol to the waste. Larger wash buffer volumes could lead to less interference caused by non-specific binding, but the amount of ligand retained on AChE-ICER will also be reduced proportionally. As for the wash buffer flow rate, three different flow rates (2, 5, 10  $\mu\text{L}/\text{min}$ ) were tested. The results showed that a lower flow rate is beneficial to enzyme-ligand binding, as the interaction time between compounds and immobilized enzyme increased. However, it will also increase the analysis time. Finally, as compromises the wash volume and the flow rate of the washing step were set at 200  $\mu\text{L}$  and 5  $\mu\text{L}/\text{min}$ , respectively.

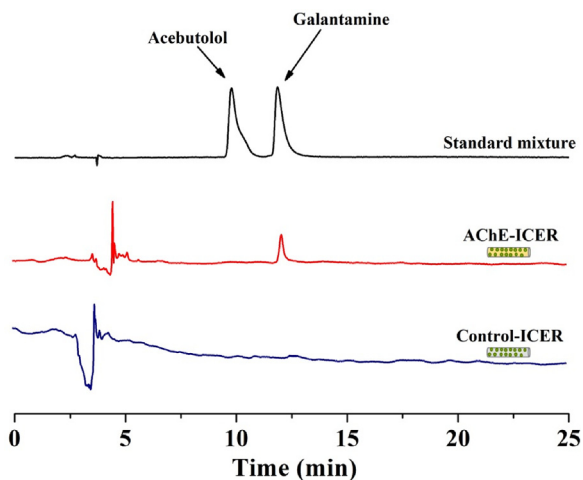
The comparative online ligand fishing platform was then evaluated using a standard solution mixture containing galantamine (AChE inhibitor) and acebutolol (non-AChE inhibitor). The denatured AChE-ICER was used as negative control to assess non-specific binding. As shown in Fig. 5, the peak of galantamine was only observed for the functional AChE-ICER as affinity SPE column for ligand fishing (red line), while acebutolol was not retained on both the functional AChE-ICER and the negative control-ICER (blue line). These results demonstrate the applicability of the platform to specifically target AChE binders in a mixture.

### 3.6. Application to plant extract

Five natural extracts, i.e. *Rhizoma coptidis*, *Rhizoma corydalis*, *Huperzia serrata*, *Lycoris radiata* and *Chrysanthemum indicum*, were reported to be rich in alkaloids or flavonoids, and their AChE



**Fig. 4.** Lineweaver-Burk reciprocal plot for ATCh (A) and dose-response inhibition curve for galantamine (B).



**Fig. 5.** LC separations of a standard mixture of acebutolol and galantamine after direct injection (black line), and after ligand fishing using AChE-ICER (red line) and after loading on the control-ICER (blue line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inhibitory activities have been reported before [29,43–45]. Therefore, they were selected and analyzed by AChE-ICER using the micro-LC-UV format as described in Section 2.5 (Table 1). The extract of *Corydalis yanhusuo* exhibited the highest AChE inhibitory

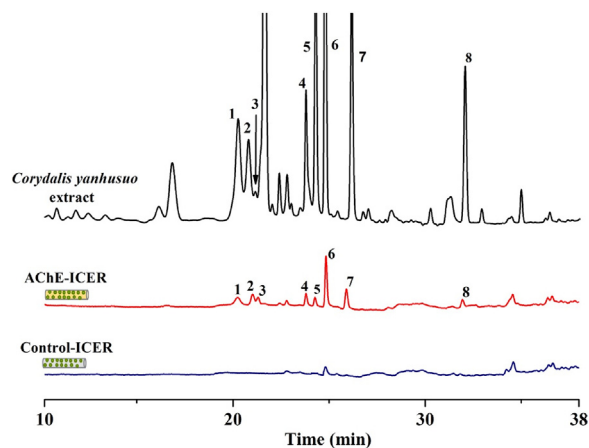
**Table 1**

AChE inhibitor analysis of natural extracts by AChE-ICER in micro-LC-UV format.

Natural extracts	Parts used	Type of extract	Inhibition (%)
<i>Chrysanthemum indicum</i>	Flower	Ethyl acetate	17.2 ± 1.2
<i>Rhizoma coptidis</i>	Root	Methanol	18.3 ± 0.7
<i>Rhizoma corydalis</i>	Tuber	Ethyl acetate	32.6 ± 4.1
<i>Huperzia serrata</i>	Whole	Ethyl acetate	19.7 ± 0.5
<i>Lycoris radiata</i>	Bulb	Methanol	17.5 ± 1.2

<sup>a</sup> Micro-LC-UV conditions were as described in Section 2.5.

<sup>b</sup> The concentration of each natural extract was 0.1 mg/mL.



**Fig. 6.** LC-MS (black trace), AChE-ICER-LC-MS (red trace) and control-ICER-LC-MS (blue trace) of the *Corydalis yanhusuo* extract. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

activity (about 32.6% inhibition rate at 0.1 mg/mL), and was selected for further studies. As the bioactive constituents contributing to this inhibitory effect are not known at this stage, the established comparative online ligand fishing platform was applied next. For this, first the separation and identification conditions for the *Corydalis yanhusuo* extract were optimized using LC-MS. Fishing and identification of potential ligands was accomplished using the complete comparative online ligand fishing platform with the optimized LC-MS protocol, which took less than 3 h overall. The system was able to fish out anti-AChE bioactive compounds from *Corydalis yanhusuo* extract and directly analyze them using online LC-MS. The analysis using a negative control-ICER revealed non-specifically interacting compounds. As shown in Fig. 6, several compounds were retained on both the AChE-ICER and control-ICER. The latter indicated potential false positives, which might be the result of non-specific binding of components to the monolith material and/or enzyme at non-functional sites. Notably, peak 6 was observed with both the AChE-ICER and control-ICER, but the peak area obtained with the control-ICER was considerably lower. Therefore, the responsible compound was still designated a potential binder. In total, eight components of *Corydalis yanhusuo* were identified as AChE binders.

Tentative assignment of eight components was performed based on their exact mass and MS/MS fragments information obtained by online LC-MS and related literatures [13,46–48]. It was then confirmed by standards. By comparing retention times ( $t_R$ ) and mass spectra of those standards with the online ligand fishing results, compounds 1–8 were identified as columbamine, jatrorrhizine, coptisine, palmatine, berberine, dehydrocorydaline, tetrahydropalmatine and corydaline, respectively (Table 2).

Inhibitory activities of the eight found compounds were further determined using the *in vitro* AChE inhibitory assay in microplate reader format. The results obtained are summarized in Table 3. It



**Table 2**  
LC/MS data of the bioactive constituents found in the *Corydalis yanhusuo* extract.

No.	$t_R$ (min)	Identification	Formula	Theoretical $m/z$	Measured $m/z$	Error (ppm)	MS/MS fragment ions ( $m/z$ )
1	20.25	Columbamine	C <sub>20</sub> H <sub>20</sub> NO <sub>4</sub>	338.1387	338.1363	-7.10	323.1112, 322.1054, 308.0892, 294.1104, 279.0872
2	21.30	Jatrorrhizine	C <sub>20</sub> H <sub>20</sub> NO <sub>4</sub>	338.1387	338.1364	-6.80	323.1111, 322.1053, 308.0895, 294.1104, 279.0872
3	21.59	Coptisine	C <sub>19</sub> H <sub>14</sub> NO <sub>4</sub>	320.0917	320.0890	-8.44	318.0738, 292.0949, 290.0795, 262.0845, 234.0896
4	23.80	Palmatine	C <sub>21</sub> H <sub>22</sub> NO <sub>4</sub>	352.1543	352.1526	-4.83	337.1264, 336.1207, 322.1053, 308.1262, 294.1107, 292.0952
5	24.27	Berberine	C <sub>20</sub> H <sub>18</sub> NO <sub>4</sub>	336.1230	336.1212	-5.36	321.0954, 320.0897, 306.0744, 292.0952, 278.0798, 275.0924
6	24.84	Dehydrocorydaline	C <sub>22</sub> H <sub>24</sub> NO <sub>4</sub>	366.1699	366.1672	-7.37	351.1418, 350.1361, 336.1206, 322.1418, 308.1259, 306.1102
7	25.90	Tetrahydropalmatine	C <sub>21</sub> H <sub>25</sub> NO <sub>4</sub>	356.1856	356.1834	-6.18	193.1039, 192.1010, 176.0696, 165.0899, 150.0664
8	31.94	Corydaline	C <sub>22</sub> H <sub>27</sub> NO <sub>4</sub>	370.2013	370.1991	-5.94	354.1676, 192.1009, 165.0901, 150.0664

**Table 3**  
Inhibition activities of AChE inhibitors found in *Corydalis yanhusuo* extract.

Compounds	Inhibition (%)	IC <sub>50</sub> (μM)
Columbamine	30.2 ± 4.6 <sup>a</sup>	11.4 ± 1.2
Jatrorrhizine	34.4 ± 4.7 <sup>a</sup>	8.1 ± 0.9
Coptisine	47.3 ± 3.3 <sup>a</sup>	4.9 ± 0.6
Palmatine	56.4 ± 3.6 <sup>a</sup>	3.8 ± 0.4
Berberine	69.7 ± 11.0 <sup>b</sup>	3.1 ± 0.6
Dehydrocorydaline	9.8 ± 3.5 <sup>a</sup>	13.0 ± 0.8
Tetrahydropalmatine	37.8 ± 5.8 <sup>b</sup>	ND
Corydaline	34.2 ± 1.5 <sup>b</sup>	ND

ND, not determined.

<sup>a</sup> Sample concentration, 5 μM.

<sup>b</sup> Sample concentration, 500 μM.

is worth noting that tetrahydropalmatine and corydaline, whose inhibition rates are lower than that of the other six identified phyto-components from 1 to 2 orders of magnitude, were retained in good amount on the AChE-ICER column. The reason is still not clear. Due to their poor solubility in phosphate buffer solution (pH 8.0), their IC<sub>50</sub> values could not be measured. The IC<sub>50</sub> values of other six compounds are less than 15 μM. These results verify that the proposed comparative online ligand fishing platform indeed is selective for AChE ligands from natural products such as TCMs.

#### 4. Conclusions

In this research, a comparative online ligand fishing platform integrating both functional and denatured monolith-based AChE-ICERs with LC-MS is presented. The label-free ligand-fishing system successfully allowed screening and identification of AChE ligands from natural products in an automated manner. Polymeric monolith based AChE-ICERs with good physicochemical properties could be prepared straightforwardly. A comparison of the retention behavior of analytes on both functional and denatured AChE-ICERs, could filter out non-specific binders avoiding false positives. Eight compounds with AChE binding affinities were identified from extract of *Corydalis yanhusuo*. Their AChE-inhibitory activities (IC<sub>50</sub>s) were confirmed by using a standard plate reader based enzymatic assay. This platform is rapid and efficient, and it can be applied as an alternative to traditional screening methods for discovering AChE-inhibiting components from TCMs [49,50].

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