



RESEARCH ARTICLE

High Throughput Enzyme Inhibitor Screening by Functionalized Magnetic Carbonaceous Microspheres and Graphene Oxide-Based MALDI-TOF-MS

Yang Liu,¹ Yan Li,² Junyan Liu,¹ Chunhui Deng,¹ Xiangmin Zhang¹¹Department of Chemistry and Institutes of Biomedical Sciences, Fudan University, Shanghai 200433, China²Department of Pharmaceutical Analysis, School of Pharmacy, Fudan University, Shanghai, China

Abstract

In this work, a high throughput methodology for screening enzyme inhibitors has been demonstrated by combining enzyme immobilized magnetic carbonaceous microspheres and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) with graphene oxide as matrix. First, model enzyme acetylcholinesterase (AChE) was immobilized onto the 3-glycidoxypropyltrimethoxysilane (GLYMO)-modified magnetic carbonaceous (MC) microspheres, displaying a high enzyme activity and stability, and also facilitating the separation of enzyme from substrate and product. The efficiency of immobilized AChE was monitored by biochemical assay, which was carried out by mixing enzyme-immobilized MC microspheres with model substrate acetylcholine (ACh), and subsequent quantitative determination of substrate ACh and product choline using graphene oxide-based MALDI-TOF-MS with no background inference. The limit of detection (LOD) for ACh was 0.25 fmol/ μ L, and excellent linearity ($R^2=0.9998$) was maintained over the range of 0.5 and 250 fmol/ μ L. Choline was quantified over the range of 0.05 and 15 pmol/ μ L, also with excellent linearity ($R^2=0.9994$) and low LOD (0.15 fmol/ μ L). Good accuracy and precision were obtained for all concentrations within the range of the standard curves. All together, eight compounds (four known AChE inhibitors and four control chemical compounds with no AChE inhibit effect) were tested with our promoted methodology, and the obtained results demonstrated that our high throughput screening methodology could be a great help to the routine enzyme inhibitor screening.

Key words: Enzyme inhibitor screening, Functionalized magnetic carbonaceous microspheres, MALDI, Graphene oxide, High throughput

Introduction

Acetylcholine (ACh) is an essential neurotransmitter, which plays a vital role in terms of memory and

learning, attention and sleep regulation [1]. Low levels of ACh are believed to be associated with pathologic conditions, such as Alzheimer's disease [2]. Since the principal function of the enzyme acetylcholinesterase (AChE) is to rapidly hydrolyze of the active neurotransmitter ACh into the inactive compounds choline and acetic acid, the inhibition of AChE activity serves as an important strategy for the treatment of Alzheimer's disease [3]. Currently, several synthetic drugs have been approved for the treatment

Electronic supplementary material The online version of this article (doi:10.1007/s13361-011-0231-8) contains supplementary material, which is available to authorized users.

Correspondence to: Xiangmin Zhang; e-mail: xmzhang@fudan.edu.cn

Received: 1 July 2011
Revised: 8 August 2011
Accepted: 9 August 2011
Published online: 27 September 2011

of Alzheimer's disease clinically [4], which all acts as AChE inhibitors to increase the amount of free ACh and improve neuron transmission [5]. Several techniques have been utilized to screen AChE inhibitors, such as colorimetric bioassay or fluorescence plate reader methodology [6]. However, these methods have some defects, such as low reproducibility or auto-fluorescence [7]. On the other hand, to address the heavy workload of large scale candidate screening, a high-throughput screening methodology is greatly needed. Very recently, mass spectrometry (MS) has been successfully utilized to monitor enzyme reaction [8]. Among which, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) based-technology attracted our attention most [9].

As a "soft" ionization technique, MALDI-TOF-MS is universally accepted as a very simple analytical approach with high speed, sensitivity, accuracy, reproducibility, and label-free readout [10, 11]. By eliminating liquid chromatography elution profiles, MALDI possesses a high-throughput potential, which greatly shortens the analysis time. Meanwhile, its higher salt and buffer tolerance characteristics can greatly simplify the sample preparation step. However, two factors severely limit the application range of MALDI-TOF-MS, which are ion signal fluctuation produced by single laser-shot as well as intense matrix background interference. From reports by others [12], the introduction of internal standards can compensate signal variations successfully. On the other hand, background interferences could be greatly reduced by utilizing proper matrices (i.e., graphene), especially for small organic molecule detection (<600 Da) [13–18]. Possessing unique electronic, optical, thermal, and mechanical properties [19], graphene oxide can be produced by oxidizing graphite, which is heavily oxygenated. Graphene oxide sheets bear hydroxyl and epoxide functional groups on basal planes, with carbonyl and carboxyl groups located at the sheet edges [20], which makes graphene oxide sheets strongly hydrophilic, easy to swell and dispersible in water [21]. Up to now, graphene oxide has been successfully utilized as a MALDI-TOF-MS matrix to detect small molecules [18, 22–24]. However, no attempt has been made to detect ACh and choline with graphene oxide before. Hence, in this work, graphene oxide as a MALDI-TOF-MS matrix was utilized to detect ACh and choline.

With the aid of MALDI-TOF-MS technology, the time duration of an enzyme inhibitor screening process could be greatly reduced. However, to further shorten this screening procedure, the control of the enzyme digestion step is also very important. Presently, immobilized enzymes have been widely utilized. Compared with conventional in-solution digestion, immobilized enzymes can greatly shorten the digestion time [25]. Recently, various beads and surface materials have been utilized for protease immobilization [26], among which magnetic microspheres have attracted increasing attention in our group [27–31]. Through immobilization, the enzyme's behavior can be improved in terms of

storage properties and pH stability. Also, by the aid of a magnetic field, these enzymes can be separated rapidly from the reaction mixture and reused [32], which can greatly facilitate the digestion process and save time.

In this work, a high throughput enzyme inhibitor screening methodology was introduced. By utilizing 3-glycidoxypropyltrimethoxysilane (GLYMO)-modified magnetic carbonaceous (MC) microspheres, the AChE enzyme could be effectively immobilized through the interaction between the epoxy groups of GLYMO and amide-functionalized groups of AChE. The performance of the immobilized enzyme was further tested by MALDI-TOF-MS after incubation with substrate ACh. Graphene oxide was utilized here to detect ACh and choline. The workflow was illustrated in Figure 1a. In addition, a mini-compound library containing eight compounds (four known AChE inhibitors and four control chemical compounds with no AChE inhibit effect) were tested, by spiking with substrate ACh and incubating with immobilized AChE, respectively.

Experimental

Chemicals and Reagents

GLYMO, R-cyano-4-hydroxy-cinnamic acid (CHCA), AChE, ACh (purity >99%), choline chloride (>99%), acetyl- β -methylcholine chloride (>98%), stable isotope choline- d_9 chloride (*N,N,N*-trimethyl- d_9 , 98% D enrichment), galanthamine hydrobromide and tacrine were all purchased from Sigma (St. Louis, MO, USA). Ferulic acid, rutin, morin, paeonol, huperzine A (Hup A), and huperzine B (Hup B) were purchased from the Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). Acetonitrile was chromatographic grade (Merk, Darmstadt, Germany). Distilled water was purified by a Milli-Q system (Milford, MA, USA). Graphene oxide was from Shanghai Boson Technology Co., Ltd. Other reagents were of analytical grade and were purchased from Shanghai Chemical Reagent Co. (Shanghai, China).

Preparation of GLYMO-Functioned MC Microspheres

The GLYMO-functioned MC microspheres with core-shell structure were synthesized in our laboratory [33]. Briefly, the MC microspheres were first synthesized through solvothermal reaction, using $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ as a single iron source and glucose as the ligand. The mixed reagents were heated at 180 °C for 6 h in a Teflon-lined stainless-steel autoclave, and then the products were obtained and washed using magnetic force. The obtained MC microspheres were refluxed with methylbenzene containing GLYMO at 80 °C for 12 h and dried at 40 °C for 24 h to obtain the GLYMO-functioned MC microspheres.

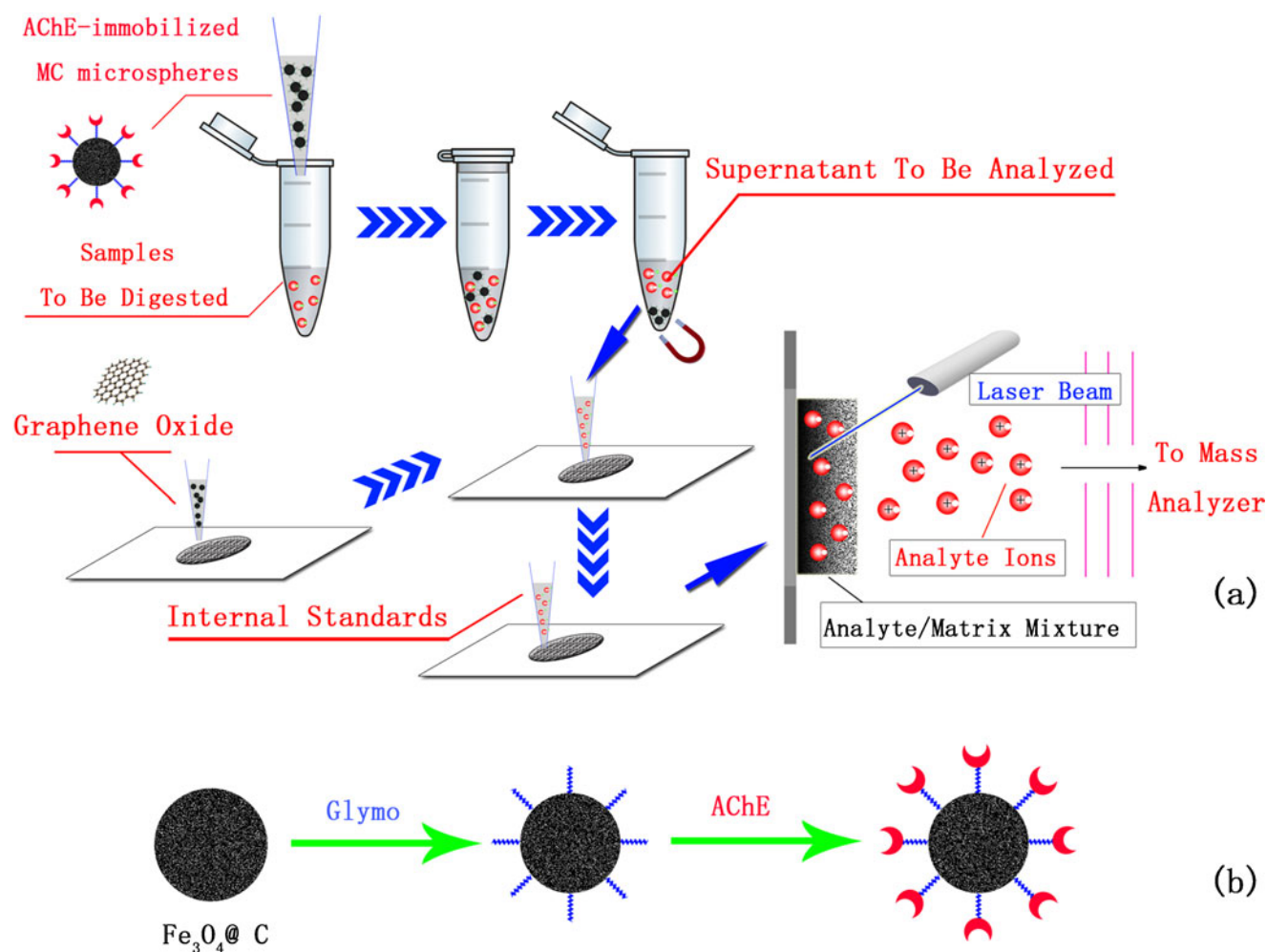


Figure 1. (a) Flow chart of the proposed enzyme inhibitor screening methodology with MC microspheres and MALDI-TOF-MS with graphene oxide as matrix. (b) Illustration of AChE immobilization onto the GLYMO-functionalized MC microspheres

Immobilization of AChE onto the GLYMO-Functioned MC Microspheres

GLYMO-functionalized MC microspheres (3 mg) were transferred to a 0.6 mL Eppendorf (EP) tube and dispersed in 200 μL 1 mg/mL AChE solution (50 mM NH_4HCO_3 as buffer, pH 7.8). The mixture was ultrasonicated for 1 min to form a homogeneous suspension (Supporting Information, Figure S1). Then the suspension was centrifuged at 37 $^\circ\text{C}$ for 1 h. After that, the supernatant solution was removed with the help of a magnet. The retained magnetic microspheres were washed three times with buffer, re-dispersed in 200 μL buffer, and stored at 4 $^\circ\text{C}$ before assay. The simple immobilization is illustrated in Figure 1b.

The UV absorption value of the supernatant solution was measured at $\lambda=280$ nm. By comparing with the ultraviolet (UV) absorption value of the AChE solution before immobilization, the amount of AChE immobilized

onto the GLYMO-functionalized MC microspheres could be calculated.

Sample Preparation

All stock solutions, standard solutions, and dilution series were prepared in 50 mM NH_4HCO_3 (pH 7.8). The stock solutions for acetylcholine, choline, acetyl- β -methylcholine chloride, and choline- d_9 were prepared at a concentration of 0.1 M. Acetyl- β -methylcholine chloride and choline- d_9 were used as internal standards. The stock solutions were used to prepare the dilution series. Both ACh and choline were analyzed in the range from 15 pmol/ μL to 0.5 fmol/ μL . Two internal standards were used at the constant final concentrations of 100 fmol/ μL for acetyl- β -methylcholine chloride and 3 pmol/ μL for choline- d_9 , separately.

The calibration standard solutions for substrate ACh and product choline in AChE inhibition assay were also prepared, by diluting the stock solutions to the concentrations of 0.25, 0.5, 1.25, 2.5, 25, 50, 125, 250, 500 fmol/ μ L for ACh and 0.025, 0.05, 0.15, 0.3, 1.5, 3.0, 7.5, 15, 30 pmol/ μ L for choline. The concentrations for two internal standards were 100 fmol/ μ L for acetyl- β -methylcholine chloride and 250 fmol/ μ L for choline- d_9 , respectively.

Matrix Preparation and MALDI Spotting

One mg of graphene oxide was dispersed as MALDI matrix in a 1 ml solution of ethanol/water (1:1, vol/vol) and then sonicated for 3 min. To achieve a homogeneous layer, the optimized three-level dried droplet preparation was carried out here. One μ L of the graphene oxide suspension was pipetted onto the MALDI target quickly. It was left in the air at room temperature to form a thin layer, and then, a 0.5 μ L solution of analyte from AChE Assay was pipetted onto the layer of matrix and left in the air for evaporation of the solvent. After that, 0.5 μ L of internal standards mixture (100 fmol/ μ L acetyl- β -methylcholine chloride and 250 fmol/ μ L choline- d_9) was spotted onto the sample crystals and left to dry completely.

MALDI-TOF-MS Analysis

Matrix assisted laser desorption/ionization mass spectra (MALDI-TOF-MS) were acquired in positive reflection mode between m/z 100 and 600 on a 5800 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA) with the Nd:YAG laser at 355 nm, a repetition rate of 200 Hz, and an acceleration voltage of 20 kV. Two thousand laser shots per spot were accumulated at 50 different spot positions. The MS/MS fragmentation was performed at the collision energy of 2 kV using air as the collision gas at a pressure of 1×10^{-6} Torr. Two thousand laser shots per precursor mass were accumulated at 50 different spot positions. The 4000 Series Explorer V3.5.3 software and the Data Explorer Software V4.8 were used for operating the mass spectrometer, as well as for data acquisition and processing.

Method Validation and Calibration Curve

Three replicate experiments were carried out to obtain each data listed in this work. A calibration curve was constructed by plotting the intensity of the analyte relative to the intensity of its corresponding internal standard on the y-axis and the analyte concentration on the x-axis. Linear regression coefficient (R^2), relative standard deviation (RSD), and accuracy were calculated by Origin 8.0 software. Accuracy was obtained by comparing the nominal values with the experimentally determined concentrations. The concentrations for each analyte were calculated from the resulting peak intensity ratios and the regression equation of the calibration curve.

AChE Assay

Briefly, the reaction mixtures of AChE-immobilized MC microspheres (20 μ L, 21 μ g) and substrate (20 μ L, 100 fmol/ μ L acetylcholine), in the presence or absence of eight tested compounds from a mini library (four known AChE inhibitors and four pending AChE inhibitor candidates), were prepared separately in 50 mM NH_4HCO_3 buffer to a final volume of 200 μ L, and incubated for 20 min at 37 $^\circ\text{C}$. The molar ratio of enzyme AChE to each inhibitor candidate was 1:1000. A magnet was used to gather and isolate the microspheres, with the supernatant waiting for further MALDI-TOF-MS analysis.

Meanwhile, for comparison, the assay was also performed by free AChE, i.e., without inhibitor. A 200 μ L mixture of 21 μ g AChE and substrate (0.1 mg/mL acetylcholine chloride) was incubated for 20 min at 37 $^\circ\text{C}$, and then, 20 μ L acetonitrile was added to terminate the reaction.

Results and Discussion

Performance Exploration of Graphene Oxide as MALDI-TOF-MS Matrix

For MALDI-TOF-MS, the commonly utilized matrices are CHCA, sinapinic acid and DHB, especially for macromolecule detection [34]. However, the performances of these matrices are not desirable when utilized to detect low molecular weight compounds (<600 Da) [35]. Strong background interferences were observed when CHCA was used as matrix [17], i.e., ACh and choline detection [12]. These interferences would greatly obscure the targeted compounds' detection. In this work, graphene oxide was utilized as a MALDI matrix to detect ACh and choline. As shown in Figure 2a, background interferences were completely reduced with graphene oxide as the matrix. Meanwhile, no cation adduct ions such as sodium adduct $[\text{M}+\text{Na}]^+$ or potassium adduct $[\text{M}+\text{K}]^+$ ions were observed.

Moreover, the detection reproducibility and sensitivity of graphene oxide matrix on positive ion mode were also investigated. Thirty sample spots were prepared with graphene oxide as the matrix, and the continuous five positive ion mass spectra were acquired from a discrete location for each spot (200 laser shots for each mass spectrum). The results are illustrated in Figure 2b, indicating that for compounds ACh (m/z 146.1) and choline (m/z 104.1), stable signal intensity and good shot-to-shot reproducibility can be achieved by utilizing graphene oxide. One of the obstacles of utilizing MALDI-TOF-MS as a reliable quantification method lies in the strong fluctuations in signal intensities. This kind of problem can be addressed by utilizing graphene oxide as a MALDI matrix to a certain degree. Meanwhile, the inhomogeneous co-crystallization of the analytes was also greatly reduced by our optimized three-level dried droplet protocol, which also helped to minimize signal intensity variations. Besides, two internal

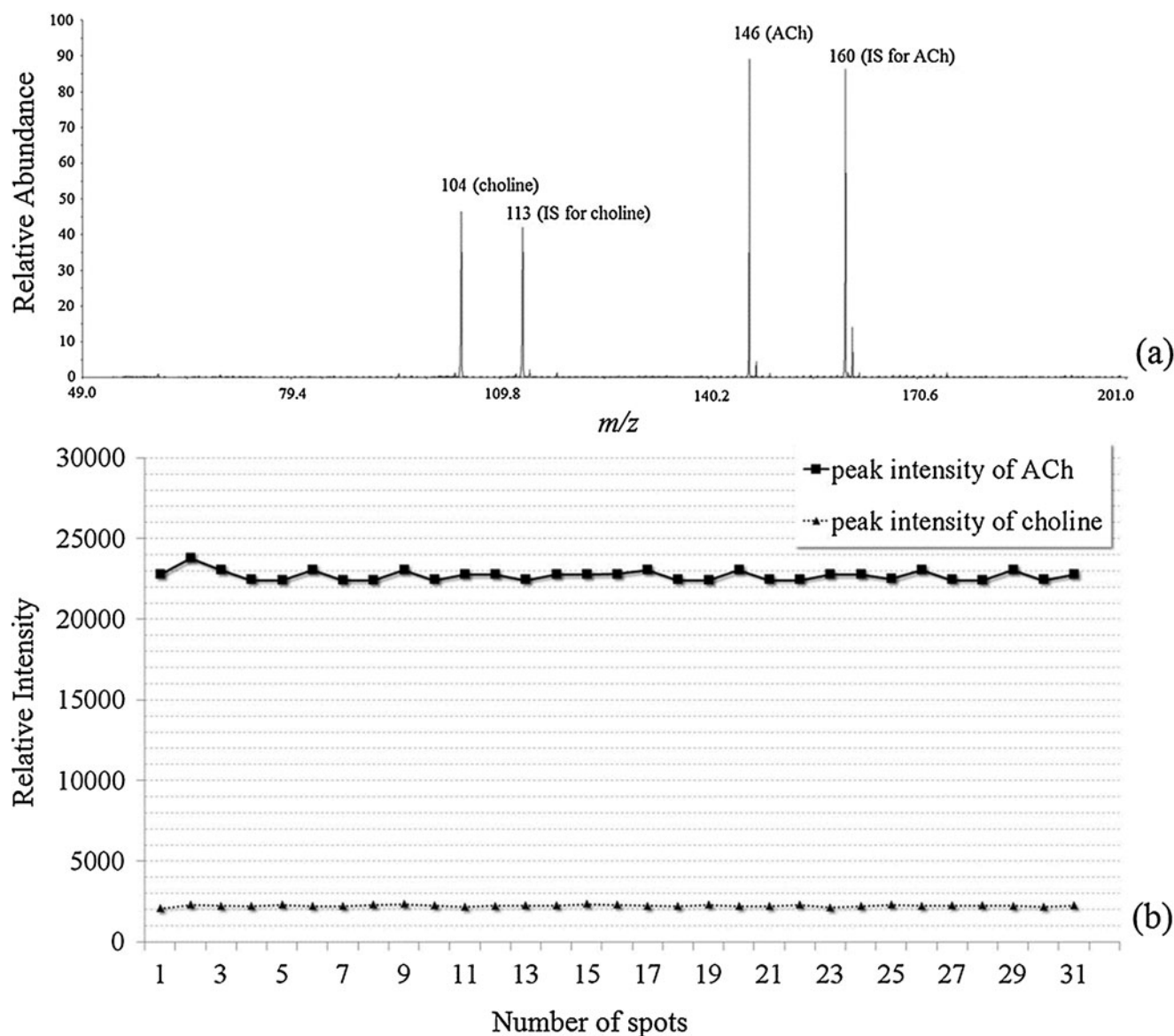


Figure 2. (a) Mass spectrum of a solution containing 100 fmol/ μ L ACh, 100 fmol/ μ L acetyl- β -methylcholin (internal standard for ACh), 250 fmol/ μ L choline, and 250 fmol/ μ L choline- d_9 (internal standard for choline) with graphene oxide as MALDI-TOF-MS matrix. (b) Comparison of peak intensities (ACh and choline) with graphene oxide as MALDI-TOF-MS matrix. The concentrations were 200 fmol/ μ L for Ach and 100 fmol/ μ L for choline, respectively

standards were simultaneously detected, which are acetyl- β -methylcholin (m/z 160, internal standard for ACh) and choline- d_9 (m/z 113.2, internal standard for choline). The introduction of these two internal standards was helpful for quantitative analysis, which could compensate for the remaining variations.

Quantitative Method Validation and Calibration Curves

Suggested by others [12], the best options for internal standards are isotopically labeled standards, which are expensive and frequently unavailable. To address this problem, structurally analogous compounds are fre-

quently utilized as a substitution. In this work, both acetyl- β -methylcholin and choline- d_9 were utilized as internal standards for ACh and choline, respectively. Their relative intensities were obtained by calculating the peak intensity ratio of targeted ions (substrate ACh and product choline) to their respective internal standards. No significant differences in RSD and accuracy between the two internal standards were noted. For ACh, the calibration curve was analyzed in the concentration range of 0.5–250 fmol/ μ L and internal standard acetyl- β -methylcholin at a constant concentration of 100 fmol/ μ L (Figure 3a). Choline was analyzed simultaneously in the concentration range of 0.05–15 pmol/ μ L, with internal standard choline- d_9 at 250 fmol/ μ L (Figure 3b).

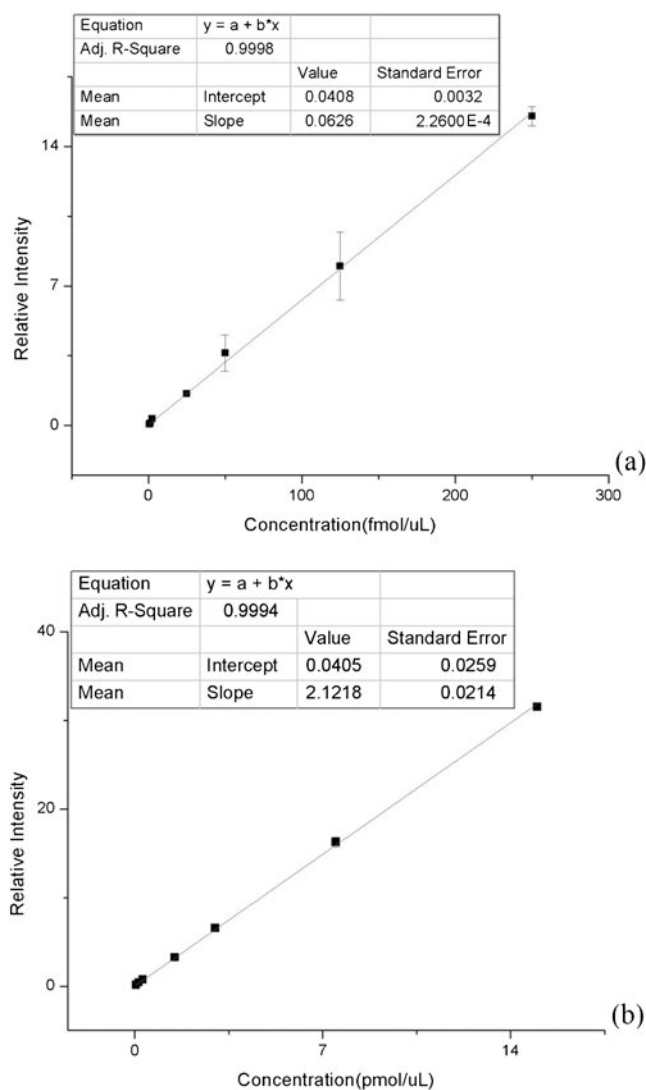


Figure 3. Calibration Curves of **(a)** substrate ACh in the concentration range of 0.5–250 fmol/ μ L and **(b)** product choline in the concentration range of 0.05–15 pmol/ μ L, with graphene oxide as MALDI-TOF-MS matrix. The error bar indicate standard deviations ($n=3$)

The RSDs and accuracies for ACh and choline are listed in Tables 1 and 2. Not surprisingly, the RSD of the concentrations measured decreased with increasing sample concentration. For example, at a concentration of 25 fmol/ μ L, the RSD was 10.28 % for ACh, while a deviation of 15.80% was witnessed at the concentration of 1.25 fmol/ μ L. The limit of detection (LOD) was 0.25fmol/ μ L for ACh and 15 fmol/ μ L for choline. The limit of quantity (LOQ) was found to be 0.5 fmol/ μ L for ACh and 50 fmol/ μ L for choline. Notably, ACh shows an excellent linearity ($R^2=0.9998$) between 0.5 and 250 fmol/ μ L. While for choline, a good precision with low RSD values and an excellent linearity ($R^2=0.9994$) were obtained in the quantified region between 0.05 and 15 pmol/ μ L. Meanwhile, in Figure 2b, the

Table 1. Precision, Accuracy, and Linearity Obtained from the Measurement of ACh with Graphene Oxide as MALDI-TOF-MS Matrix. Acetyl- β -methylcholin was Utilized as Internal Standard at a Constant Concentration of 100 fmol/ μ L

ACh		
Conc (fmol/ μ L)	RSD %	Accuracy (% error)
250	0.28	-0.60
125	0.54	2.30
50	8.25	-2.77
25	10.28	8.96
2.5	12.86	-7.76
1.25	15.80	3.34
0.5	4.51	-1.36
Linearity (R^2)	0.9998	

reproducible peak intensities data of ACh and choline were mainly used for illustrating the stability of each concentration data spot. However, within the calibration curves, the peak intensity of the ion signal did not have a good linear relationship at varied concentrations. Thus, two internal standards were used and the quantification curves were obtained by calculating the intensity ratio of the peak signal. To investigate the necessity of utilizing these two internal standards, the calibration curves of these two compounds without using internal standards were obtained (Supporting Information, Figure S2). The linear dynamic ranges of ACh and choline were narrower than those obtained with internal standards, and their linear regression coefficients became worse.

ACh has been detected by various methods, such as liquid chromatography/electrochemical (LC-EC) method [36–38], liquid chromatography/electrospray ionization/mass spectrometry (LC-ESI-MS) [8, 39–42], liquid chromatography/atmospheric pressure chemical ionization/mass spectrometry (LC-APCI-MS) [43], as well as MALDI-TOF-MS with CHCA as the matrix [12]. The performances of these methods have been listed in detail on Table 3. Compared with these performances, our new high throughput MALDI-TOF-MS methodology could achieve a lower LOD than the traditional LC-EC applications, with no need for chromatographic separation or desalting steps. Meanwhile, by utilizing graphene oxide as the MALDI matrix, a

Table 2. Precision, Accuracy, and Linearity Obtained from the Measurement of Choline with Graphene Oxide as MALDI-TOF-MS Matrix. Choline- d_3 was Utilized as Internal Standard at a Constant Concentration of 250 fmol/ μ L

Choline		
Conc (pmol/ μ L)	RSD %	Accuracy (% error)
15	1.22	-0.59
7.5	3.39	2.27
3	6.41	6.82
1.5	3.41	3.80
0.3	10.22	11.59
0.15	14.84	19.67
0.05	26.64	-19.04
Linearity (R^2)	0.9994	

Table 3. Performances of Current Reported Methods in Terms of ACh Detection

Detection method	LOD (fmol/ μ L)	LOD linearity (R^2)	LOQ (fmol/ μ L)	LOQ linearity (R^2)	Reference
LC-EC	10	0.999	-	-	[28]
	-	-	1	>0.990	[29]
	5	-	10	0.999	[30]
LC/ESI/MS	-	-	-	-	[8]
	1.4	0.9983	-	-	[31]
	1	0.9792	5	-	[32]
	0.2	-	1	0.999	[33]
	1	-	-	-	[34]
LC/APCI/MS	-	-	1.5	0.986	[35]
MALDI-TOF-MS (CHCA as matrix)	0.3	-	1	0.9996	[12]

clean MS profile with no background interferences could be obtained, with a low LOD comparable to that of LC-MS or other MALDI-TOF-MS (CHCA as matrix) methods. In addition, in our previous work [8], ESI-MS was utilized to analyze ACh and choline. As known to us all, high throughput screening is very important for enzyme inhibitor screening. Compared with ESI-MS, MALDI-MS is a high throughput instrument. Hence, finding a suitable matrix for MALDI-MS is of vital importance. In this work, graphene oxide was demonstrated to be a very good choice as MALDI matrix to analyze ACh and choline, which enables our developed screening method to be high throughput.

Preparation and Characterization of GLYMO-Functionalized MC Microspheres

GLYMO-functionalized MC microspheres were prepared by following our previous methods [44, 45]. The two-step hydrothermal reactions and one carbonization reaction enable a large-scale synthesis, which is simple and economic. According to our previous work [33], the transmission electron microscopy (TEM) image of the MC microspheres demonstrates their satisfactory uniformity with a mean diameter of 300 nm. Remarkably, the thick carbonaceous shell not only provides magnetite microspheres with nonmetallic surface for further surface modification, but also protects the enzyme from contact with iron oxide. And by choosing GLYMO as the derivative reagent for MC microspheres, better immobilization ratio and ability of the enzyme for structural recognition can be realized, compared with other reactive “spacer” groups such as diol, aldehyde, and tresyl groups [46, 47]. The conjugation chemistry between the enzyme and GLYMO is mainly accomplished by the epoxy chemistry. The chemical reaction between the epoxy group on GLYMO and free amino groups on enzyme usually occurs at the pH 7–8. Epoxy chemistry is an appropriate system for developing easy protocols, due to

its stability at neutral pH values, wet conditions, and reactivity with several nucleophilic groups (i.e., free amino groups). In our previous work [33], during the procedure of immobilizing trypsin to GLYMO functionalized MC microspheres, strong chemical bonds were formed. In addition, Fourier transform infrared (FT-IR) spectroscopy characterization further confirmed the successful modification of the MC microspheres by GLYMO molecules, which is very suitable for enzyme immobilization [33].

Immobilization of AChE onto GLYMO-Functionalized MC Microspheres

AChE was immobilized to the GLYMO-functionalized MC microspheres in a one-step reaction. The immobilization ability of GLYMO-functionalized MC microspheres for AChE was determined by measuring the UV absorption of the supernatant enzyme solution. Usually, enzyme immobilization relies largely on experimental conditions, such as pH, time, and reaction temperature. Hence, efforts have been made to optimize the immobilization conditions. The amount of immobilized AChE differed when the pH was varied from 5.8 (acetate buffer), 6.8 (phosphate buffer), 7.8 (ammonium bicarbonate buffer), 8.8 (phosphate buffer) to 9.8 (sodium bicarbonate buffer). As shown in Figure 4a, the higher pH value (pH > 7.8) resulted in similar reaction efficiency between the epoxy group of GLYMO and the amide functionalized groups of the enzyme. When pH value was < 7.8, weak cross link reactions were witnessed between GLYMO and enzyme, which can be proven by the low amount of immobilized AChE. Therefore, a 50 mM ammonium bicarbonate buffer (pH 7.8) was recommended here. Moreover, the proper immobilization time was investigated. No further improvement was observed when the time duration was more than 60 min, which may be related to the saturated epoxy groups on GLYMO-functionalized MC microspheres (Figure 4b). The temperature dependency of this immobilization was also investigated. As shown in Figure 4c, the immobilization efficiency still increased slightly with a temperature higher than 37 °C, which indicated that a higher temperature can improve this cross link reaction. A higher temperature (>37 °C) may result in the irreversible structural destruction to enzyme AChE. Hence, this immobilization procedure was conducted at 37 °C for 60 min.

According to the UV absorption measurements (calibration data not shown), the amount of AChE immobilized onto the MC microspheres was about 66 μ g/mg. In addition, to testify the necessity of using GLYMO to immobilize enzyme, control experiments have been carried out to explore the non-specific binding of the enzyme to the beads. The same AChE immobilization procedure was performed using beads without GLYMO. The UV absorption was used to determine the immobilization effect. No obvious changes of UV signal were observed of the supernatant solution. Due to the high sensitivity of the MALDI MS, a certain

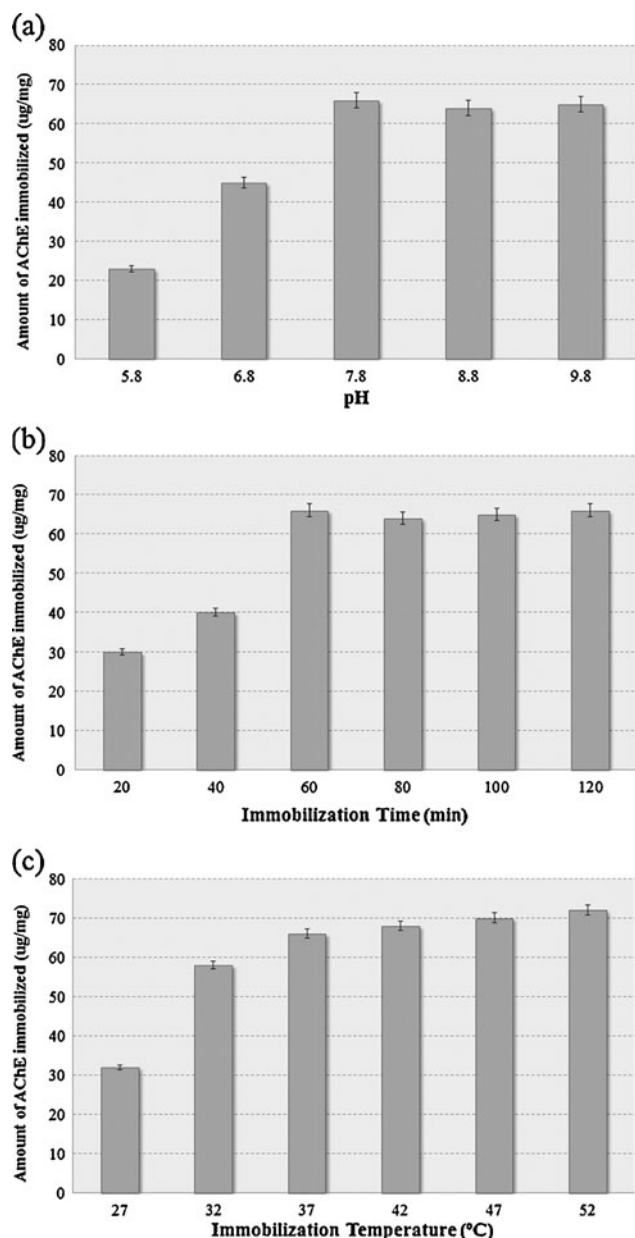


Figure 4. Optimizing immobilization conditions of enzyme AChE onto GLYMO-functionalized MC microspheres: **(a)** Influence of pH value on the immobilization efficiency; **(b)** Influence of reaction time on the immobilization efficiency; **(c)** Influence of reaction temperature on the immobilization efficiency. The error bar indicate standard deviations ($n=3$)

hydrolyzing results were observed (the result is shown in Supporting Information, Figure S3). We assumed that as there existed lots of hydroxyl groups and carboxyl groups on the beads without GLYMO, the electrostatic interactions would possibly occur between the beads and amino acids of the enzyme. However, to achieve an effective enzyme immobilization, this kind of electrostatic interaction force was comparatively weak, which makes it meaningful to introduce GLYMO.

Performance of AChE-Immobilized MC Microspheres

The AChE assay was carried out as illustrated in Figure 1a. Briefly, the AChE-immobilized MC microspheres were dispersed in buffer solution and incubated with substrate ACh under 37 °C. With the help of a magnet, the reactant can be quickly separated from the MC microspheres. Meanwhile, to realize a high throughput evaluation, MALDI-TOF-MS was utilized here, with graphene oxide as the matrix, to detect the amount of reduced substrate ACh and released product choline. Figure 5a shows the MS spectrum before digestion. Only peaks for the substrate ACh (m/z 146.1) and the two internal standards could be found. Meanwhile, Figure 5b shows a typical MALDI-TOF-MS spectrum after 20 min digestion. The complete disappearance of the substrate ACh peak (m/z 146.1) and the appearance of the choline peak (m/z 104.1) fully demonstrated the existence of the hydrolysis reaction. In the presence of AChE, ACh (m/z 146.1) can be rapidly hydrolyzed into product choline (m/z 104.1) and acetic acid. No particular requirement of separation technique was required, which enabled our methodology to be rapid, easy, economic, and high throughput.

The hydrolysis condition of immobilized enzyme AChE was optimized as to the hydrolysis time, incubation temperature as well as molar ratios of substrate ACh to enzyme AChE (for more detailed information, please see Supporting Information). As shown in Supporting Information, Figure S4, the optimum hydrolysis efficiency could be achieved after a 20 min hydrolysis at 37 °C, with a molar ratio of 192:1 (ACh to AChE).

AChE Inhibitor Screening

The key mechanism of Alzheimer's disease treatment drugs lies in their ability to inhibit AChE. In this article, a mini-compound library was employed to evaluate our new methodology, which contained four known AChE inhibitors (galanthamine, tacrine, Hup A, and Hup B) [8, 48] and four control chemical compounds extracted from natural herbs (ferulic acid, rutin, morin, paeonol) with no AChE inhibit effect. Each compound was tested at the molar ratio of 1:1000 (enzyme AChE:each tested compound). As shown in Figure 1c, the experiment was carried out as follows: AChE microspheres were firstly dispersed in 50 mM NH_4HCO_3 buffer, and then incubated with substrate ACh (m/z 146.1) and each compound from the mini library at 37 °C for 20 min, followed by rapid isolation with a magnet; and finally the supernatant was measured by MALDI-TOF-MS with the graphene oxide matrix. If AChE inhibition took effect, the ratio of product choline (m/z 104.1) to internal standard choline- d_9 (m/z 113.2) would decrease, while the ratio of substrate ACh (m/z 146.1) to internal standard acetyl- β -methylcholine (m/z 160.1) would increase. Each compound's inhibition efficiency could be calculated

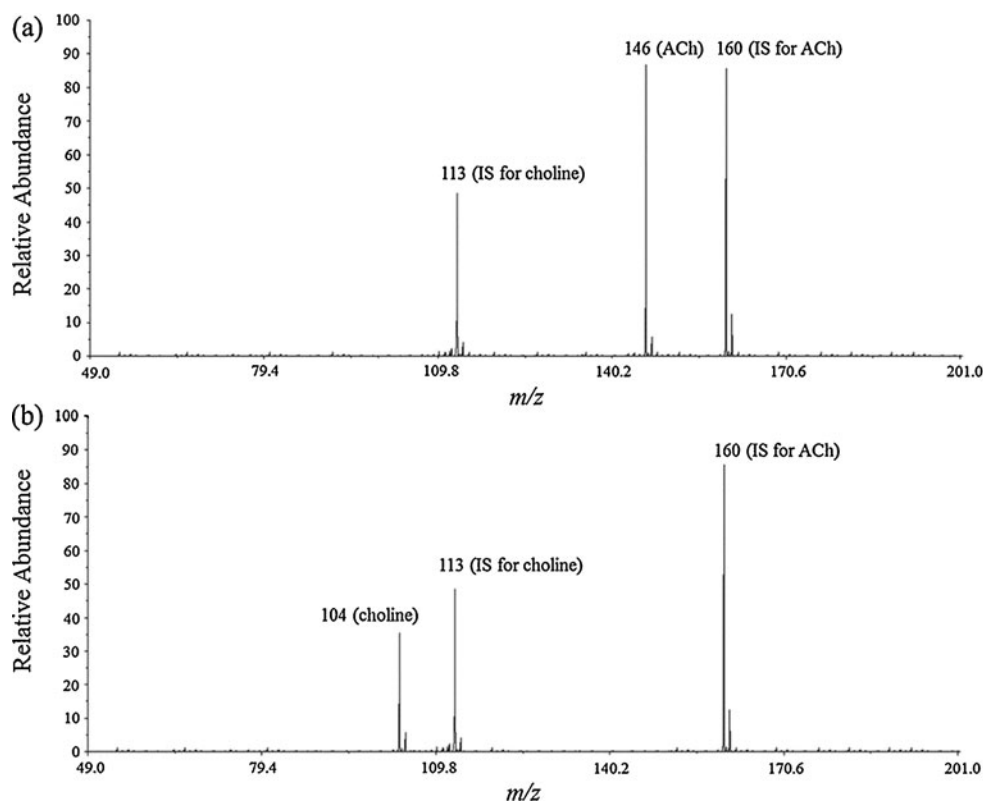


Figure 5. MALDI-TOF-MS profiles of ACh before digestion (a) and after 20 min digestion at 37 °C (b), with acetyl- β -methylcholine (m/z 160.1) as the internal standard for ACh (m/z 146.1), and choline- d_9 (m/z 113.2) as the internal standard for choline (m/z 104.1)

according to the following equations:

$$\text{Inhibition Efficiency (\%)} = \frac{K_{\text{blank}} - K_{\text{sample}}}{K_{\text{blank}}} \times 100\% \quad (1)$$

$$K_{\text{blank}} = \text{IR}_{\text{blankend}} - \text{IR}_{\text{blankinitial}} \quad (2)$$

$$K_{\text{sample}} = \text{IR}_{\text{sample end}} - \text{IR}_{\text{sample initial}} \quad (3)$$

$$\text{IR} = \frac{\text{Peak Intensity of ACh (or choline)}}{\text{Peak Intensity of acetyl-}\beta\text{-methylchol (or choline-d}_9\text{)}} \quad (4)$$

IR stands for peak intensity ratio of substrate ACh to internal standard acetyl- β -methylcholine (or product choline to internal standard choline- d_9). The value of $\text{IR}_{\text{blank initial}}$ was obtained at the beginning of the AChE assay with no addition of any tested compounds from mini library, and the value of $\text{IR}_{\text{blank end}}$ was obtained at the end of this AChE

assay, in the absence of eight tested compounds. Values for $\text{IR}_{\text{sample initial}}$ and $\text{IR}_{\text{sample end}}$ were acquired respectively at the beginning and the end of AChE assay, with the presence of one tested compound from the mini library. Each compound's inhibition efficiency was determined in this manner (Table 4).

Among the four known AChE inhibitors, galanthamine was demonstrated to be the strongest AChE inhibitor with an inhibition efficiency of 98.2% at the molar ratio of 1:1000 (enzyme AChE:galanthamine). The effect of this enzyme

Table 4. The Inhibition Efficiencies of Eight Compounds from Mini-library, which Contain Four Known AChE Inhibitors (Galanthamine, Tacrine, Hup A, and Hup B) and Four Control Chemical Compounds Extracted from Natural Herbs (Ferulic Acid, Rutin, Morin, Paeonol) with No AChE Inhibit Effect. The Molar Ratio of Enzyme AChE to each Tested Compound was 1:1000

Compound from mini-library	Inhibition efficiency (%)
Galanthamine	98.2
Tacrine	97.9
Hup A	73.4
Hup B	76.8
Ferulic Acid	0
Rutin	0
Morin	0
Paeonol	0

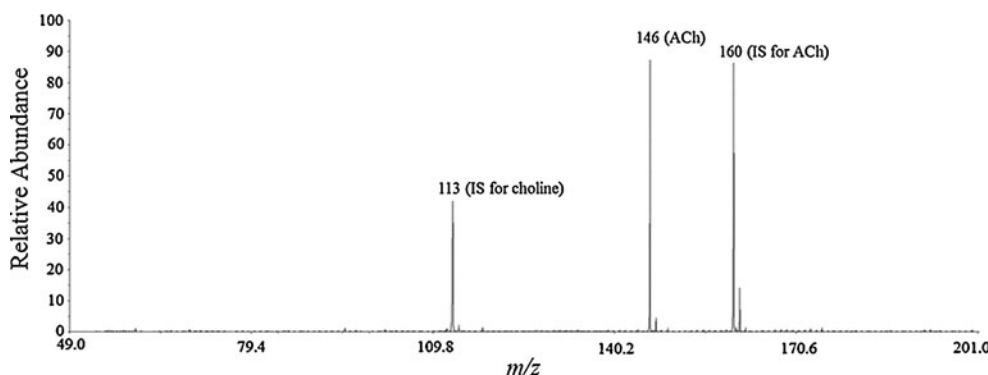


Figure 6. The mass spectrum of the effect of enzyme inhibitor galanthamine. The molar ratio of enzyme AChE to galanthamine was 1:1000

inhibitor galanthamine was shown in Figure 6. Tacrine ranked the second with an inhibition efficiency of 97.9% at the same molar ratio. While for Hup A and Hup B, at a molar ratio of 1:1000, the inhibition efficiencies are 73.4% for Hup A and 76.8% for Hup B. According to our previous work [8], product choline's peak completely vanished when the molar ratio is about 1:2000 (enzyme AChE:Hup A or enzyme AChE:Hup B). The current study provides a more precise and visual result as to these two drugs' inhibition ability, respectively. Besides, no inhibition effect was found among the four control chemical compounds (ferulic acid, rutin, morin, paeonol), which was in good accordance with these four chemicals' characteristics. By screening of these four control chemical compounds, the reliability of our developed high throughput methodology was proved to be valid.

In summary, for screening of the present mini compound library, a rapid but valid analysis was realized. By utilizing functionalized MC microspheres and MALDI-TOF-MS with graphene oxide as matrix, our promoted high throughput methodology would do a lot favor for large scale AChE inhibitor candidates screening.

Conclusions

In this work, a high throughput methodology for screening AChE inhibitor was developed. GLYMO-functionalized magnetic carbonaceous microspheres were synthesized and coated with enzyme AChE. This enzyme-immobilized material possesses excellent characteristics, such as easy isolation, cost saving, high efficiency, and recyclability, and has great potential in a large-scale production. In addition, graphene oxide was utilized to quantify ACh and choline. Apart from reducing background interferences, many other advantages are also presented here, such as simple sample preparation, low LOD and LOQ, improved shot-to-shot reproducibility, and excellent linearity. By combining AChE immobilized MC microspheres and MALDI-TOF-MS with graphene oxide as the matrix, our methodology has the potential for rapid and sensitive measurements useful for pharmaceutical research.

Acknowledgment

The authors acknowledge support for this work by the National Basic Research Priorities Program (project: 2007CB914100), the National Science Foundation of China (project: 21027002), the Research Fund for the Doctoral Program of Higher Education of China (no. 20100071120053), Shanghai Municipal Natural Science Foundation (no. 11ZR1403200), and Shanghai Leading Academic Discipline Project (B109).

References

- Wessler, I., Kirkpatrick, C.J., Racke, K.: Non-Neuronal Acetylcholine, a Locally Acting Molecule, Widely Distributed in Biological Systems: Expression and Function in Humans. *Pharmacol. Ther.* **77**, 59–79 (1998)
- Felician, O., Sandson, T.A.: The Neurobiology and Pharmacotherapy of Alzheimer's Disease. *J. Neuropsychiatry Clin. Neurosci.* **11**, 19–31 (1999)
- Greenblatt, H.M., Dvir, H., Silman, I., Sussman, J.L.: Acetylcholinesterase: A Multifaceted Target for Structure-Based Drug Design of Anticholinesterase Agents for the Treatment of Alzheimer's Disease. *J. Mol. Neurosci.* **20**, 369–383 (2003)
- Zangara, A.: The Psychopharmacology of Huperzine A: An Alkaloid with Cognitive Enhancing and Neuroprotective Properties of Interest in the Treatment of Alzheimer's Disease. *Pharmacol. Biochem. Behav.* **75**, 675–686 (2003)
- Winkler, J., Thal, L.J., Gage, F.H., Fisher, L.J.: Cholinergic Strategies for Alzheimer's Disease. *J. Mol. Med.* **76**, 555–567 (1998)
- Funeri, D.P., Eppinger, J., Denizot, L., Miyake, M., Miyake, J.: Enzyme Family-Specific and Activity-Based Screening of Chemical Libraries Using Enzyme Microarrays. *Nat. Biotechnol.* **23**, 622–627 (2005)
- Grepin, C., Pernelle, C.: High-throughput screening—Evolution of Homogeneous Time Resolved Fluorescence (HTRF) Technology for HTS. *Drug Discov. Today* **5**, 212–214 (2000)
- Hu, F., Zhang, H., Lin, H., Deng, C., Zhang, X.: Enzyme Inhibitor Screening by Electrospray Mass Spectrometry with Immobilized Enzyme on Magnetic Silica Microspheres. *J. Am. Soc. Mass Spectrom.* **19**, 865–873 (2008)
- Hu, L., Jiang, G., Xu, S., Pan, C., Zou, H.: Monitoring Enzyme Reaction and Screening Enzyme Inhibitor Based on MALDI-TOF-MS Platform with a Matrix of Oxidized Carbon Nanotubes. *J. Am. Soc. Mass Spectrom.* **17**, 1616–1619 (2006)
- Karas, M., Hillenkamp, F.: Laser Desorption Ionization of Proteins with Molecular Masses Exceeding 10,000 Daltons. *Anal. Chem.* **60**, 2299–2301 (1988)
- Porta, T., Grivet, C., Knochenmuss, R., Varesio, E., Hopfgartner, G.: Alternative CHCA-Based Matrices for the Analysis of Low Molecular Weight Compounds by UV-MALDI-Tandem Mass Spectrometry. *J. Mass Spectrom.* **46**, 144–152 (2011)
- Persike, M., Zimmermann, M., Klein, J., Karas, M.: Quantitative Determination of Acetylcholine and Choline in Microdialysis Samples by MALDI-TOF MS. *Anal. Chem.* **82**, 922–929 (2010)

13. Cohen, L.H., Gusev, A.I.: Small Molecule Analysis by MALDI Mass Spectrometry. *Anal. Bioanal. Chem.* **373**, 571–586 (2002)
14. Dong, X., Cheng, J., Li, J., Wang, Y.: Graphene as a Novel Matrix for the Analysis of Small Molecules by MALDI-TOF MS. *Anal. Chem.* **82**, 6208–6214 (2010)
15. Luo, Y.B., Shi, Z.G., Gao, Q.A., Feng, Y.Q.: Magnetic Retrieval of Graphene: Extraction of Sulfonamide Antibiotics from Environmental Water Samples. *J. Chromatogr. A* **1218**, 1353–1358 (2011)
16. Liu, Q., Shi, J.B., Sun, J.T., Wang, T., Zeng, L.X., Jiang, G.B.: Graphene and Graphene Oxide Sheets Supported on Silica as Versatile and High-Performance Adsorbents for Solid-Phase Extraction. *Angew. Chem. Int. Ed.* **50**, 5913–5917 (2011)
17. Lu, M.H., Lai, Y.Q., Chen, G.N., Cai, Z.W.: Matrix Interference-Free Method for the Analysis of Small Molecules by Using Negative Ion Laser Desorption/Ionization on Graphene Flakes. *Anal. Chem.* **83**, 3161–3169 (2011)
18. Gulbakan, B., Yasun, E., Shukoor, M.I., Zhu, Z., You, M.X., Tan, X.H., Sanchez, H., Powell, D.H., Dai, H.J., Tan, W.H.: A Dual Platform for Selective Analyte Enrichment and Ionization in Mass Spectrometry Using Aptamer-Conjugated Graphene Oxide. *J. Am. Chem. Soc.* **132**, 17408–17410 (2010)
19. Wang, Z.J., Zhou, X.Z., Zhang, J., Boey, F., Zhang, H.: Direct Electrochemical Reduction of Single-Layer Graphene Oxide and Subsequent Functionalization with Glucose Oxidase. *J. Phys. Chem. C* **113**, 14071–14075 (2009)
20. He, H.Y., Klinowski, J., Forster, M., Lerf, A.: A New Structural Model for Graphite Oxide. *Chem. Phys. Lett.* **287**, 53–56 (1998)
21. Szabo, T., Szeri, A., Dekany, I.: Composite Graphitic Nanolayers Prepared by Self-Assembly Between Finely Dispersed Graphite Oxide and a Cationic Polymer. *Carbon* **43**, 87–94 (2005)
22. Liu, Y., Liu, J.Y., Yin, P., Gao, M.X., Deng, C.H., Zhang, X.M.: High Throughput Identification of Components from Traditional Chinese Medicine Herbs by Utilizing Graphene or Graphene Oxide as MALDI-TOF-MS matrix. *J. Mass Spectrom.* **46**, 804–815 (2011)
23. Zhou, X., Wei, Y., He, Q., Boey, F., Zhang, Q., Zhang, H.: Reduced Graphene Oxide Films Used as Matrix of MALDI-TOF-MS for Detection of Octachlorodibenzo-p-Dioxin. *Chem. Commun.* **46**, 6974–6976 (2010)
24. Kim, Y.K., Na, H.K., Kwack, S.J., Ryoo, S.R., Lee, Y., Hong, S., Jeong, Y., Min, D.H.: Synergistic Effect of Graphene Oxide/MWCNT Films in Laser Desorption/Ionization Mass Spectrometry of Small Molecules and Tissue Imaging. *ACS Nano* **5**, 4550–4561 (2011)
25. Babiak, P., Reymond, J.L.: A High-Throughput, Low-Volume Enzyme Assay on Solid Support. *Anal. Chem.* **77**, 373–377 (2005)
26. Gao, J., Xu, J., Locascio, L.E., Lee, C.S.: Integrated Microfluidic System Enabling Protein Digestion, Peptide Separation, and Protein Identification. *Anal. Chem.* **73**, 2648–2655 (2001)
27. Li, Y., Leng, T., Lin, H., Deng, C., Xu, X., Yao, N., Yang, P., Zhang, X.: Preparation of Fe₃O₄@ZrO₂ Core-shell Microspheres as Affinity Probes for Selective Enrichment and Direct Determination of Phosphopeptides Using Matrix-Assisted Laser Desorption Ionization Mass Spectrometry. *J. Proteome Res.* **6**, 4498–4510 (2007)
28. Li, Y., Lin, H., Deng, C., Yang, P., Zhang, X.: Highly Selective and Rapid Enrichment of Phosphorylated Peptides Using Gallium Oxide-Coated Magnetic Microspheres for MALDI-TOF-MS and Nano-LC-ESI-MS/MS/MS Analysis. *Proteomics* **8**, 238–249 (2008)
29. Li, Y., Liu, Y., Tang, J., Lin, H., Yao, N., Shen, X., Deng, C., Yang, P., Zhang, X.: Fe₃O₄@Al₂O₃ Magnetic Core-Shell Microspheres for Rapid and Highly Specific Capture of Phosphopeptides with Mass Spectrometry Analysis. *J. Chromatogr. A* **1172**, 57–71 (2007)
30. Li, Y., Wu, J., Qi, D., Xu, X., Deng, C., Yang, P., Zhang, X.: Novel Approach for the Synthesis of Fe₃O₄@TiO₂ Core-Shell Microspheres and Their Application to the Highly Specific Capture of Phosphopeptides for MALDI-TOF MS Analysis. *Chem. Commun.* **5**, 564–566 (2008)
31. Li, Y., Qi, D., Deng, C., Yang, P., Zhang, X.: Cerium Ion-Chelated Magnetic Silica Microspheres for Enrichment and Direct Determination of Phosphopeptides by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry. *J. Proteome Res.* **7**, 1767–1777 (2008)
32. Sanjay, G., Sugunan, S.: Glucoamylase Immobilized on Montmorillonite: Synthesis, Characterization, and Starch Hydrolysis Activity in a Fixed Bed Reactor. *Catal. Commun.* **6**, 525–530 (2005)
33. Yao, G., Qi, D., Deng, C., Zhang, X.: Functionalized Magnetic Carbonaceous Microspheres for Trypsin Immobilization and the Application to Fast Proteolysis. *J. Chromatogr. A* **1215**, 82–91 (2008)
34. Mank, M., Stahl, B., Boehm, G.: 2,5-Dihydroxybenzoic Acid Butylamine and Other Ionic Liquid Matrixes for Enhanced MALDI-MS Analysis of Biomolecules. *Anal. Chem.* **76**, 2938–2950 (2004)
35. Sleno, L., Volmer, D.A.: Some Fundamental and Technical Aspects of the Quantitative Analysis of Pharmaceutical Drugs by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **19**, 1928–1936 (2005)
36. Huang, T., Yang, L., Gitzen, J., Kissinger, P.T., Vreeke, M., Heller, A.: Detection of Basal Acetylcholine in Rat Brain Microdialysate. *J. Chromatogr. B Biomed. Appl.* **670**, 323–327 (1995)
37. de Boer, P., Westerink, B.H., Horn, A.S.: The effect of Acetylcholinesterase Inhibition on the Release of Acetylcholine from the Striatum In Vivo: Interaction with Autoreceptor Responses. *Neurosci. Lett.* **116**, 357–360 (1990)
38. Tsai, T.R., Cham, T.M., Chen, K.C., Chen, C.F., Tsai, T.H.: Determination of Acetylcholine by On-Line Microdialysis Coupled with Pre- and Post-Microbore Column Enzyme Reactors with Electrochemical Detection. *J. Chromatogr. B Biomed. Appl.* **678**, 151–155 (1996)
39. Zhu, Y., Wong, P.S., Cregor, M., Gitzen, J.F., Coury, L.A., Kissinger, P.T.: In Vivo Microdialysis and Reverse Phase Ion Pair Liquid Chromatography/Tandem Mass Spectrometry for the Determination and Identification of Acetylcholine and Related Compounds in Rat Brain. *Rapid Commun. Mass Spectrom.* **14**, 1695–1700 (2000)
40. Hows, M.E., Organ, A.J., Murray, S., Dawson, L.A., Foxton, R., Heidbreder, C., Hughes, Z.A., Lacroix, L., Shah, A.J.: High-Performance Liquid Chromatography/Tandem Mass Spectrometry Assay for the Rapid High Sensitivity mMeasurement of Basal Acetylcholine from Microdialysates. *J. Neurosci. Methods* **121**, 33–39 (2002)
41. Uutela, P., Reinila, R., Piepponen, P., Ketola, R.A., Kostainen, R.: Analysis of Acetylcholine and Choline in microdialysis Samples by Liquid Chromatography/Tandem Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **19**, 2950–2956 (2005)
42. Lacroix, L.P., Ceolin, L., Zocchi, A., Varnier, G., Garzotti, M., Curcuruto, O., Heidbreder, C.A.: Selective Dopamine D₃ Receptor Antagonists Enhance Cortical Acetylcholine Levels Measured with High-Performance Liquid Chromatography/Tandem Mass Spectrometry Without Anti-Cholinesterases. *J. Neurosci. Methods* **157**, 25–31 (2006)
43. Keski-Rahkonen, P., Lehtonen, M., Ihalainen, J., Sarajarvi, T., Auriola, S.: Quantitative Determination of Acetylcholine in Microdialysis Samples Using Liquid Chromatography/Atmospheric Pressure Spray Ionization Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **21**, 2933–2943 (2007)
44. Xu, X.Q., Deng, C.H., Gao, M.X., Yu, W.J., Yang, P.Y., Zhang, X.M.: Synthesis of mMagnetic Microspheres with Immobilized Metal ions for Enrichment and Direct Determination of Phosphopeptides by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry. *Adv. Mater.* **18**, 3289 (2006)
45. Li, Y., Yan, B., Deng, C., Yu, W., Xu, X., Yang, P., Zhang, X.: Efficient On-Chip Proteolysis System Based on Functionalized Magnetic Silica Microspheres. *Proteomics* **7**, 2330–2339 (2007)
46. Shinkai, M., Ito, A.: Functional Magnetic Particles for Medical Application. *Adv. Biochem. Eng. Biotech.* **91**, 191–220 (2004)
47. Marle, I., Karlsson, A., Pettersson, C.: Separation of Enantiomers Using α -Chymotrypsin-Silica as a Chiral Stationary Phase. *J. Chromatogr.* **604**, 185–196 (1992)
48. Blunder, M., Hurkes, N., Spirk, S., List, M., Pietschnig, R.: Silanetriols as in vitro inhibitors for AChE. *Bioorg. Med. Chem. Lett.* **21**, 363–365 (2011)