

Acetylcholinesterase immobilized on modified magnetic beads as a tool for screening a compound library

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Abstract Acetylcholinesterase (AChE) from *Electrophorus electricus* was immobilized on the surface of amino-modified magnetic beads (AChE-MB), and its activity evaluated by the quantification of acetylcholine hydrolysis. A reference mixture composed of AChE binders (galanthamine and a probe coumarin, $K_i=0.031\pm 0.010$ μM) and non-binders (ketamine and propranolol) was used to probe the fishing assay. The performance of the bioconjugation assay was demonstrated with a library of 12 reference coumarins from which two ligands were directly identified by LC-MS/MS in a single assay, demonstrating the usefulness of this approach.

Keywords Binding affinity assay · Acetylcholinesterase inhibitor · Label-free assay · Fishing assay · Ligand fishing · Coumarin library

Introduction

Affinity or bioconjugation based screening assays has been employed as an approach to investigate and identify ligands in complex mixtures [1–7]. The development of bioreactors containing bioactive targets (proteins, receptors, enzymes, lipids, etc.) should preserve the macromolecule conformation and function. To this, a support that allows the biomolecule

immobilization and reduces non-specific interactions is necessary [8–12].

Modified magnetic beads have an advantage, as they provide a stable support for protein immobilization and have been used successfully for ligand isolation for several proteins, c.f. HSA/BSA [4, 13, 14], acetylcholinesterase [7], xanthine oxidase [15], SIRT6 [2, 3], etc. In the magnetic bioconjugation assay, protein-coated beads are immersed directly into the mixture. Any compounds with an affinity for the immobilized protein will be retained while non-binders will remain in the solution. The bound compounds can then be eluted for their identification [4, 5, 7, 16–18].

Herein we report on an inhibitor label-free bioconjugation assay based on AChE-coated magnetic beads for screening ligand libraries. A coumarin library of twelve compounds (Fig. 1) was used, two of which were retained, characterized by LC-MS/MS and identified as AChE ligands.

This procedure assimilates the HTS (High Throughput Screening) and FBLD (Fragment-Based Lead Discovery) assay features, supporting the screening of ligand mixtures with low, high or non-affinity for the enzyme.

Experimental

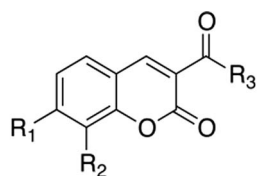
Chemicals

Acetylcholine iodide (ACh), choline iodide (Ch), galanthamine bromide (GAL), nicotine, ketamine, acetylcholinesterase from *Electrophorus electricus* type VI-S, (3-aminopropyl) triethoxysilane (APTS) 99 %, ammonium acetate, tris(hydroxymethyl)aminoethane and all chemical materials used during the immobilization procedure were purchased from Sigma-Aldrich (www.sigmaaldrich.com). Propranolol was donated by AstraZeneca do Brazil (www).

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Coumarin	R ₁	R ₂	R ₃
1	HO	H	OH
2	H ₃ CO	H	OH
3	H	OH	OH
4	H ₃ CO	H	OCH ₂ CH ₃
5	H ₃ CO	H	
6	H	H	
7	HO		OCH ₂ CH ₃
8	HO		OCH ₂ CH ₃
9	HO		OCH ₂ CH ₃
10	HO	H	
11		H	OCH ₂ CH ₃
12	H	H	

Fig. 1 Chemical structures of the reference coumarin library

astrazeneca.com.br). BcMag™ amine-terminated magnetic beads were purchased from Bioclone (www.bioclone.us). Methanol and acetonitrile were HPLC grade and the water used for all experiments was deionized in a Millipore Milli-Q system. The mobile phases were prepared daily. The chromatography columns were Atlantis® HILIC (3 μm, 2.1 × 100 mm), purchased from Waters (www.waters.com), and Kinetex® Biphenyl (2.6 μm, 2.1 × 100 mm), generously donated by Allchrom (Phenomenex representative - www.phenomenex.com). The coumarin library (Fig. 1) was prepared as previously described [19].

Chromatography system and conditions

The LC system (Shimadzu, Kyoto, Japan) used consisted of two LC-20 AD pumps, a SIL 20A autosampler with a 50 μL

loop, a DGU-20A5 degasser and a CBM-20A interface. The LC system was coupled to an Esquire 6000 IT mass spectrometer (Bruker Daltonics, GmbH, Bremen, Germany) equipped with an ESI source, operating in both ionization modes (positive and negative). Data acquisition was carried out using the Bruker Daltonics data analysis software. All LC analyses were carried out at room temperature (±20 °C).

Preparation of AChE-modified magnetic beads

The AChE was immobilized through the N-terminus on the surface of modified magnetic beads using either an aqueous pyridine solution [10 mM, pH 6.0 adjusted with a 1 M NaOH] or phosphate buffer, resulting in AChE-MB (1- using pyridine) and AChE-MB (2 – using phosphate buffer), respectively. The procedure for preparing AChE-MB –1 is based in a previously reported protocol [7] while for AChE-MB –2, 25 mg of magnetic beads was washed three times with 1.0 mL of phosphate buffer [100 mM, pH 7.0]. Using a homemade manual magnetic separator, the supernatant was discarded and the beads were suspended in 1.0 mL of phosphate buffer [100 mM, pH 7.0] containing 5 % glutaraldehyde and shaken, at 4.0 °C, for 3.0 h. After magnetic separation, the beads were washed three more times with 1.0 mL of phosphate buffer [100 mM, pH 7.0], followed by the addition of 0.40 mg of AChE in 500 μL of phosphate buffer [100 mM, pH 7.0]. The reaction was left for 16 h at 4.0 °C with gentle rotation. The supernatant was discarded and the beads were washed three times with 500 μL of Tris–HCl [100 mM, pH 8.0] and stored at this buffer at 4.0 °C.

The AChE-MBs activities were measured by the reaction with ACh at 200 μM, prepared in ammonium acetate solution (15 mM, pH 8.0). The supernatants were collected after 120 s, using a magnetic separator. The acetylcholine hydrolysis product, Ch ([M]⁺ 104 m/z) was quantified in the positive ion mode (ESI+) by multiple reaction-monitoring (MRM) data acquisition, in accordance with a previously reported LC-MS/MS method [20], this time using an uncoated capillary (30 × 0.010 cm, d.i.) to avoid enzyme interactions. The MB used as control was submitted to the same immobilization procedure, but replacing glutaraldehyde and the enzyme solution for phosphate buffer [100 mM, pH 7.0].

Validation of ligand fishing assay

The AChE-MB-2 was suspended in 500 μL of an ammonium acetate buffer [15 mM, pH 8.0] containing 100 nM of the reference compounds (ketamine, propranolol, galanthamine and a probe coumarin (Fig. 2, 11: ethyl 2-oxo-7-(2-(piperidin-1-yl)ethoxy)-2H-chromene-3-carboxylate), in a mixture. The tube was mixed by vigorously shaking for 30 s, set for 90 s and placed into the magnetic separator for 120 s. The supernatant (S-1) was collected, and the AChE-MB

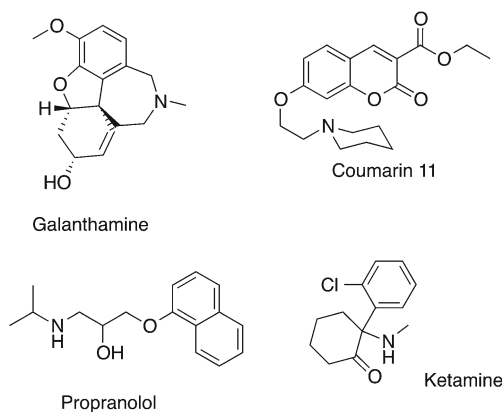


Fig. 2 Chemical structures of reference compounds used to validate the bioconjugation assay

was washed twice with 500 μL of ammonium acetate [15 mM; pH 8.0] by vigorously shaking for 10 s, placing into the magnetic separator for 120 s.

The AChE-MB was then suspended in the extraction solution (500 μL of ammonium acetate [15 mM, pH 8.0] containing 20 % of methanol and 1826 μM ACh) and was shaken at 20 rpm with a RevolverTM Rotator (Labnet) at 23 $^{\circ}\text{C}$ for 15 min. The supernatant (S-2) was collected and the AChE-MB was washed twice. To maintain a similar ratio of organic to aqueous, 100 μL of methanol was added to S-1 and, 100 μL of ammonium acetate buffer [15 mM, pH 8.0] was added to S-2. Nicotine at 20 μM was used as internal standard. For plotting the results, the ratios of the areas for the compounds present in S-1 and S-2 over the internal standard were calculated. A control experiment was carried out using the non-coated MB.

The samples (S-1 and S-2) were injected in the LC-MS/MS system using an Atlantis[®] HILIC as the analytical column with ACN:ammonium acetate buffer [15 mM, pH 6.0] (84:16, v/v) as mobile phase at 200 $\mu\text{L}\cdot\text{min}^{-1}$ from which 100 $\mu\text{L}\cdot\text{min}^{-1}$ was sent to MS by a splitting adapter. The MS parameters were set, at 7.5 $\text{L}\cdot\text{min}^{-1}$ for the drying gas flow, 30.0 psi for the nebulizer pressure, 335 $^{\circ}\text{C}$ for the drying gas temperature, 4352 V for the capillary voltage, 34.5 V for the skimmer voltage and 132 V for the Cap. exit voltage. The protonated molecular ion $[\text{M}+\text{H}]^{+}$ used to identify each of the tested compounds was: galanthamine (m/z 288), coumarin derivative (m/z 346), propranolol (m/z 260), ketamine (m/z 238) and nicotine (m/z 163). The multiple reaction-monitoring (MRM) was carried out for the acquisition in the positive ion mode.

Ligand fishing of a coumarin library

Resolution of the coumarins library was achieved with a LC-MS/MS method using an Kinetex[®] biphenyl analytical column with the following conditions: flow rate 0.2 $\text{mL}\cdot\text{min}^{-1}$ and injection volume 5 μL , with the mobile phase consisting of 0.1 % aqueous formic acid (v/v) (A) and methanol with

0.1 % formic acid (B). The gradient elution was: 35–75 % (B) in 30 min, maintaining 75 % (B) for 5 min, with the total run time equal to 35 min. The MS parameters were set at 7.5 $\text{L}\cdot\text{min}^{-1}$ for the drying gas flow, 30.0 psi for the nebulizer pressure and 335 $^{\circ}\text{C}$ the drying gas temperature. Scans from 100.0 to 450 m/z were carried out in both the negative and positive ionization modes.

The coumarin library (Fig. 1), at 100 $\text{ng}\cdot\text{mL}^{-1}$ of each compound, was used for the bioconjugation assay. For that, either AChE-MBs or the control MB were suspended in the coumarin mixture and then, the supernatants, S-1 and S-2 (for the AChE-MB and for the control assay) were collected. The analyses of each collected supernatant were carried out by injecting 5 μL to the LC-MS/MS system. The total ion chromatogram was used to the acquisition while the extracted ion chromatogram was used to identify (m/z of molecular ion and fragments) the coumarins in each sample.

Results

Validation of the screening assay

The quantification of the produced choline (≈ 72 μM) by the AChE-MB-1 and AChE-MB-2 demonstrate that the immobilized enzyme retained its activity toward its natural substrate, with no significant difference in the concentration of produced choline. Thus, all further experiments were carried out with AChE-MB -2.

The reference AChE binders (galanthamine and the probe coumarin, $K_i=0.031\pm 0.010$ μM) and non-binders (ketamine and propranolol), Fig. 2, were used as a mixture to validate the bioconjugation assay. As expected, ketamine and propranolol was present with a higher area in S -1 than in S -2, demonstrating that the immobilized enzyme did not retain the non-binders. Furthermore, galanthamine and the probe coumarin, known binders, although also present in some cases in S-1, were predominantly present in S-2 (Fig. 3). This demonstrates

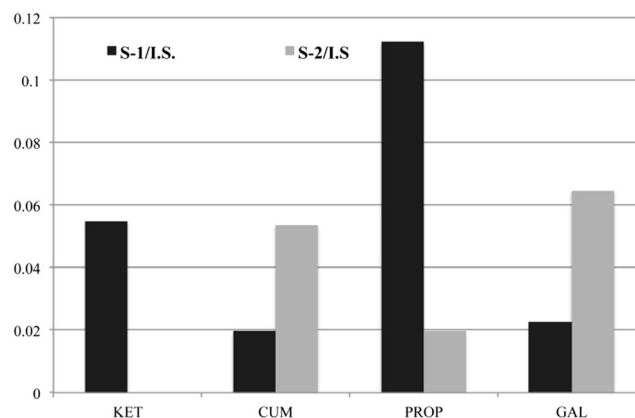


Fig. 3 The area ratio values (supernatant/internal standard) in the validation of bioconjugation assay for AChE-MB

the necessity of determining the levels of ligands in both S1 and S2, as anything present less than 15 % in S-2 should not be considered a binder. The experiment was repeated with non-coated magnetic beads and all compounds tested were present in the control S-1 in a percentage higher than 83 %, with no compound found in the control S-2, with the exception of propranol, which was present at 10 %.

Screening of a coumarin library

The versatility of bioconjugation assays with AChE-MB was demonstrated with a reference coumarin library (Fig. 1) [20]. Herein, a LC-MS/MS separation method using a fused core biphenyl column was used to identify the fished coumarins from the mixture.

The supernatants of the AChE-MB assay S-1 and S-2 were analyzed and their results compared with controls (Fig. 4). The analyses were carried out in both, negative and positive ionization modes. Out of the 12 coumarins, only Coumarin 11 and Coumarin 12 were fished by the AChE-MB and found in S-2 at a ratio over 80 % when compared to S-2 of the control MB. Coumarin 11 is the reference coumarin used to probe the bioconjugation experiment (Fig. 3) and coumarin 12 has a K_i of 13.8 ± 1.1 μM [20].

Discussion

In a classical screening assay for AChE ligands, the enzymatic reaction product is carried out in solution with acetylthiocholine as substrate and the product is determined spectrophotometrically [21]. Some drawbacks of these methods are easily noticed such as false-positive results, the inability of enzyme reuse and high costs.

Zonal chromatography is well established for screening AChE ligands [11, 20, 22, 23]. In these approach, the ligands are screened individually and the enzymatic catalysis product, choline [20] or thiocholine [22, 23] are measured.

Aiming to screen plant extracts and mixture of synthetic compounds, AChE coated on magnetic beads was

envisaged as a part of a ligand screening assay which would allow the bioconjugated ligands to be identified by tandem mass spectrometry. In this approach, the ligands based on their enzyme affinity are fished out of the mixture and the structural elucidation of the isolated binders easily carried out.

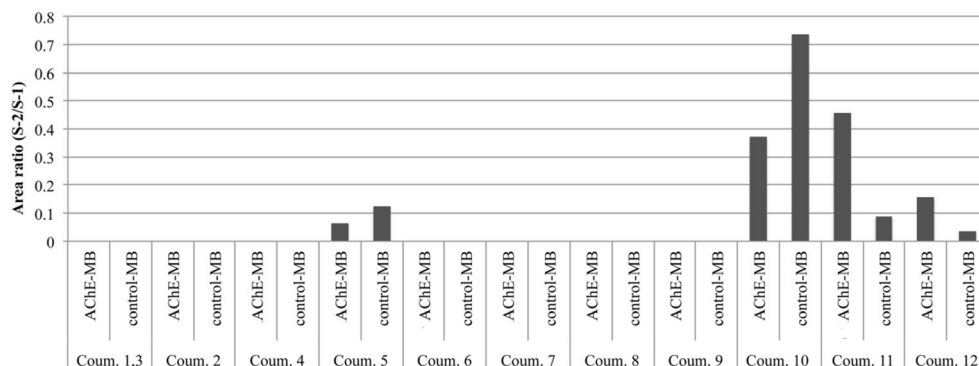
The results presented on Fig. 3 show that the fishing capacity of AChE-MB can be related to the enzyme affinity and the K_i of the ligands, with the probe coumarin (11, $K_i=0.031 \pm 0.010$ μM) being retained more strongly than galanthamine ($K_i=1.06 \pm 0.274$ μM) [20]. Although the binders were also present in S-2 in the control experiments this is of no concern since they were fished out in values higher than 80 % by the AChE-MB.

For orthogonal validation of a mixture assay, the developed approach was used to fish ligands in a mixture of coumarins. The proof of principle was established with the identification of two coumarins with affinity for the AChE being fished out of a mixture of 12 in accordance with their inhibitory capacity (Fig. 4). Coumarin 11 (%I=98, $\text{IC}_{50}=0.36 \pm 0.010$ μM and $K_i=0.031 \pm 0.010$ μM) [20], was predominantly present in S-2, followed by coumarin 12 (%I=84.0, $\text{IC}_{50}=12.6 \pm 1.07$ and $K_i=13.8 \pm 1.10$) [20]. Coumarins 5 (%I=36) and 10 (%I=23) [20] were mainly present in S-2 of the control experiment and, they appeared also in minor percentage at S-2 of the AChE-MB assay, indicating that they had non-specific interaction with the magnetic particle and not with the enzyme.

Conclusions

The ligand screening assay for AChE herein reported has significant advantages over existing systems based on enzyme activity. It is a label-free assay and furthermore can be readily adapted to the screening of any synthetic or natural library. The combination of the developed bioconjugate assay with ion trap mass spectrometry allows for the direct determination of the active compounds from complex mixtures. The target identification of the bioconjugated ligands by LC-MS/MS

Fig. 4 Supernatants area ratio: S-2/S-1 for AChE-MB and control-MB in the bioconjugation assay with the reference coumarin library



makes of this approach a reliably way to characterize low affinity hits in a simple five steps fishing experiment.

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