Electronic Supplementary Information

Inhibitory effect of nucleotides on acetylcholine esterase activity and its microflow-based actuation in blood plasma

Akshi Deshwal, Arshdeep Kaur Gill, Surajmal Nain, Debabrata Patra, Subhabrata Maiti*

Department of Chemical Sciences, Indian Institute of Science Education and Research (IISER) Mohali, Knowledge City, Manauli 140306, India.

E-mail: smaiti@iisermohali.ac.in

Table of Contents

1.1 Materials and methods:

All commercially available reagents were used without further any purification. Acetylcholinesterase from *Electrophorus electricus* (electric eel) and Choline Oxidase from Alcaligenes sp was procured from Sigma. Phosphatidylcholin (PC) lipids were procured from Avanti polar lipids. Human Plasma, Acetylthiocholine iodide (ATCI), Acetylcholine chloride (ATCL), 5,5'-Dithiobis (2-nitrobenzoic acid), Polyethylene imide and Sulfate latex particles (5µm) were purchased from Sigma. Horseradish Peroxidase (HRP) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were procured from Sisco Research laboratory (SRL). Mono-N-dodocyl phosphate, 2-ethyl hexyl phosphate, Bis 2-ethyl hexyl phosphate and Paraoxon were also purchased from Sigma. Sodium dodecyl sulfonate (SDS) was purchased from Sigma. DC/GEN/ Sylgard 184 (PDMS elastomer kit) was purchased from Kevin Electrochem. Adenosine monophosphate (AMP), Adenosine diphosphate (ADP), Adenosine triphosphate (ATP), Cyclic Adenosine Monophosphate (cAMP), Beta NADP, and Guanidium Hydrochloride (GDNHCl) were purchased from Sisco Research Laboratory (SRL). HEPES and Tris HCl buffer were purchased from SRL. Solvents like Ethanol, acetonitrile was purchased from Rankem and SRL respectively. Sulfuric acid and Hydrogen peroxide were procured from SRL.

UV-Vis studies were performed using Varian Cary 60 (Agilent technologies) spectrophotometer. Total reaction volume in the cuvette was fixed at 1 ml and cuvette of path length 1 cm was used for the entire kinetic study. All measurements have been performed at 25 ^{o}C.

Fluorescence measurements were performed using Cary Eclipse Fluorescence Spectro-fluorometer.

The optical and fluorescence microscopic images were collected using Zeiss Axis Observer 7 microscope having AxioCam 503 Mono 3 Mega pixel with ZEN 2 software.

An inverted optical microscope (Olympus IX73) was used to record all videos with a halogen lamp (100 W).

The Dynamic Light Scattering (DLS) data was recorded on Horiba Scientific Nanoparticle Analyzer SZ-100V2.

Circular Dichroism (CD) Measurements were performed using Biologic MOS 500 using a 1 mm path length quartz cell. The concentration of Acetylcholinesterase used for the CD measurement was 12 units per ml. The spectra were recorded with a scan range of 200-280 nm. All measurements were recorded in triplicate. Calculation of α-helix and β-sheet content were performed using BeStSel (Beta Structure Selection) method.[1]

1.2 Preparation of enzyme immobilised glass slides:

Enzyme immobilisation on glass slides was performed in the same way as given in literature. [2] The glass slides were first thoroughly washed thrice with acetone, isopropyl alcohol followed by Millipore water. After that the slides were rinsed in Piranha solution (A solution of conc. Sulfuric acid and Hydrogen peroxide) for 45 mins, washed with Millipore water and dried by blowing nitrogen gas over them. In the meantime, PDMS was spread over petri dish and kept in oven at 70^{O}C for 1 hr for its better settlement. Then the PDMS pieces comparable to glass slides were cut and a hole of diameter 4.8 mm was made into them. The pieces were placed onto the dried glass slides and then slides were dipped first in 1 wt % PEI (polyethyleneimine) solution called primer then in enzyme solution and after that in 0.1 wt % PEI solution and lastly again in enzyme solution. After each set of dipping, the slides were washed by dipping into the water or buffer solution depending upon in which the stock of polymer or enzyme was made. In this multilayer film of enzyme was made. Herein, we prepared two Bilayers (2BL) of enzyme. The thickness of the formed film was measured through ellipsometer. In the same way, Plasma immobilisation was also performed on the glass slides. The only difference was Plasma solution was used for dipping slides in place of enzyme solution.

1.3 Preparation of Vesicles bound with Acetylcholinesterase:

Vesicles were prepared by using thin film hydration method reported in literature.[3] 1.5 mg of PC lipids were dissolved in 1 ml of chloroform. The formed solution was evaporated under high vacuum at 30 °C for 1 hr and then dried overnight under vacuum to obtain a dry thin film. The film was rehydrated using 1 ml 5 units Acetylcholinesterase (AChE) in 5 mM HEPES buffer (pH = 7) under nitrogen atmosphere for making enzyme bound vesicles. To remove unbound acetylcholinesterase the solution was filtered with 300K polysulphone membrane under centrifugation rate of 1500 rpm for 15 minutes. Then washing was performed with buffer along with centrifugation to ensure complete removal of unbound AChE. To check removal of unbound AChE, absorbance spectra of filtrate was recorded. For our experimental purpose, concentration of AChE-tagged in vesicles is 4.52 units per ml and 10mU/ml was the final concentration used for kinetic investigation.

1.4 Preparation of FITC Tagged Acetylcholinesterase

FITC tagged acetylcholinesterase was prepared as given in literature [4]. 1 mg/ml FITC was dissolved in DMSO and then 100 µl of this was mixed with 5 units/ml of acetylcholinesterase. The solution was kept under dark at 4 C for 5 to 6 hrs. After that the ongoing reaction of tagging was quenched by addition of 50 mM of ammonium chloride and the mixture was kept for more 2 hrs. Then the reaction mixture was passed through a column made up of Sephadex G-25 and the formed conjugate was purified through column with 5 mM HEPES buffer pH: 7. The eluted

samples were collected and different vials and there UV absorption spectra was recorded in which peak at 280 nm signifies the FITC-AChE peak and at 495 nm FITC peak. Some of the amount of tagged enzyme was used for making vesicles in order to measure their anisotropy.

1.5 Tryptophan fluorescence studies with nucleotides

Tryptophan fluorescence studies was performed with 0.5 mM of nucleotides namely AMP, ADP, ATP and cAMP with a final concentration of AChE 6 units/ml in a quartz cuvette of 1 cm pathlength. The excitation wavelength was 290 nm and emission spectra were recorded in between 310 nm – 500 nm. The emission wavelength 340 nm was used for further analysis of nucleotide effect on enzyme fluorescence. The excitation and emission slit width were 10 and 10 nm respectively.

1.6 Tertiary structure studies

The above studies were performed using fluorimeter in presence of 0.5 mM Nucleotides (AMP, ADP, ATP and cAMP) with varying concentration of guanidinium hydrochloride (GDNCl) from 0 to 6 M. The final concentration of enzyme was 6 units/ml. The excitation wavelength 280 nm was used and emission spectrum was recorded between 300 nm to 500 nm. A quartz cuvette of 1 cm pathlength was used. The excitation and emission slit width were 10 and 10 nm respectively.

1.7 Measurement of anisotropy of Acetylcholinesterase:

The steady state anisotropy measurements of acetylcholinesterase (AChE) and AChE vesicles (both tagged and untagged) were performed on an LS 55 luminescence spectrometer from Perkin Elmer. Tryptophan steady state anisotropy in case of untagged AChE was measured at excitation wavelength 280 nm and emission at 340 nm with excitation and emission band pass 5 and 7 nm respectively. In case of FITC tagged AChE, steady state anisotropy was measured with the following parameters: λ_{ex}: 480 nm and λ_{em}: 525 nm with excitation and emission bandpass 1.5 and 2 nm respectively. The final concentration of enzyme and FITC was 5 unit per ml and 3 µM respectively.

1.8 Measurement of Acetylcholinesterase catalytic activity:

In Buffer: The catalytic activity measurements of enzyme AChE and vesicle bound AChE were performed using Ellman's method using acetylthiocholine iodide as substrate. 10 milli units per ml AChE along with varying concentration of substrate from 1 µM to 1000 µM and fixed concentration of DTNB i.e., 100 µM for recording enzyme's catalytic activity in buffer.

Also in buffer, Choline Oxidase method was also used in order to study catalytic activity of acetylcholinesterase. In this method three enzymes were used namely acetylcholinesterase which converts acetylcholine chloride into choline. Choline converts into betaine aldehyde and

hydrogen peroxide in presence of choline oxidase. The formation of hydrogen peroxide was detected with the help of HRP (horseradish peroxidase) in presence of ABTS. The concentration of enzymes was as followed: [AChE]: 50 mUnit/ml, [ChOx]: 50 mUnit/ml, [HRP]: 0.1 nM and substrates concentration were: [Acetylcholine chloride]: 2 mM, [ABTS]: 1 mM.

AChE's catalytic activity was also determined in presence of all nucleotides (AMP, ADP, ATP, cAMP & UTP) and alkyl phosphates (Mono-N-dodecyl phosphate, 2-ethyl hexyl phosphate, Bis 2-ethyl hexyl phosphate & Paraoxon) in buffer. This kinetic investigation was carried out using Ellman's method with fixed concentration of substrate i.e., 100 µM and two different concentration (100 & 500 µM) of nucleotides and alkyl phosphates were used.

In Human Plasma: The plasma itself contained AChE, thus 0.1 wt % Human Plasma was used for catalytic activity measurements. 100 µM acetylthiocholine iodide and 100 µM DTNB were used for kinetic investigation. Herein also two different concentration of nucleotides and paraoxon were used. In both medium whether buffer or Plasma, kinetic activity was measured by formation of chromogenic compound from acetylthiocholine and DTNB that gave absorbance at 436 nm UV spectrometer. Concentration of product was calculated using molar extinction coefficient of TNB^{2-} at pH 7 as 11000 $M^{-1}cm^{-1}$.[5]

1.9 Molecular Docking Studies:

Molecular docking studies were performed with the help of Auto dock tools 1.5.6. We used the crystallographic structure of native Acetylcholinesterase from Torpedo Californica from Protein Databank (PDB accession no. 2 ACE). In the docking experiments the protein was considered rigid and all the ligands including substrate and all nucleotides were employed flexibility. The grid box in case of substrate was centred on the active site of the enzyme whereas in case of all other ligands the blind docking was performed. The 3D structure of all the ligands were downloaded from PubChem and converted into pdbqt format using Auto dock during docking. Each ligand with receptor or protein was analysed using software Biodiscovery Studio.

1.10 Flow Studies:

The flow based micropump studies were carried out in hybridisation chamber of dimensions: length: 1 cm, width: 1cm, height: 1.8 mm using 5 µm polystyrene sulphate particles as tracer particles via an inverted optical microscope (Olympus IX73) having a halogen lamp (100 W). The sample in the chamber was excited through a 10X objective. Both enzyme immobilised slides and plasma (0.05 wt %) ones were analysed via putting substrate solution in the hybridisation chamber in absence and in presence of all nucleotides. Each of the video was analysed via software called Tracker and approx. 25 tracer particles were chosen for calculating speed for each of the video.

2. Ellman's Assay

In order to study the acetylcholinesterase kinetics, Ellman's method was used to determine the amount of product formed during reaction. In this method substrate Acetylthiocholine was used with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) which led to formation of yellow product 2-nitro-5-thiobenzoate (NTB) and monitored spectrophotometrically.

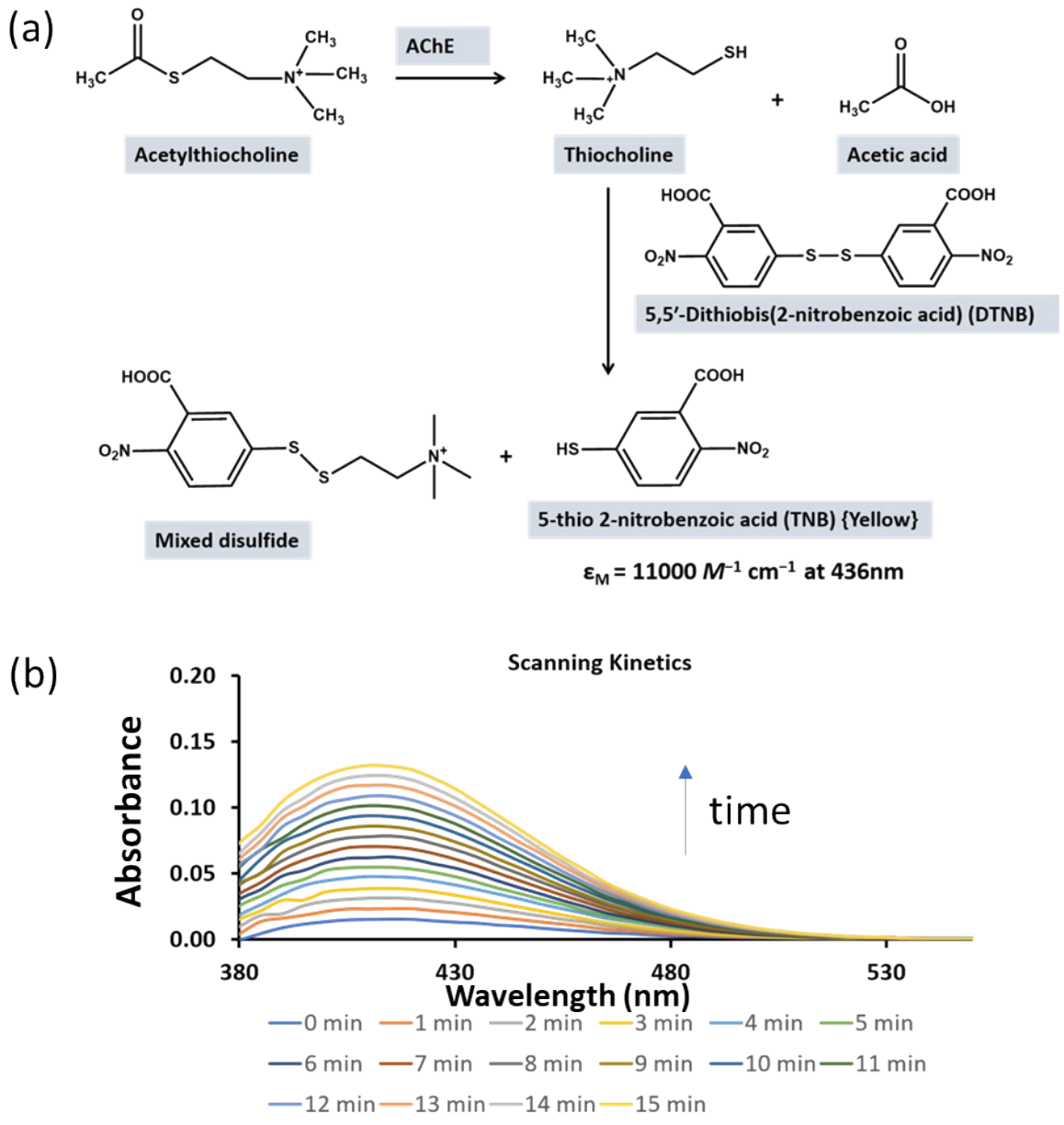


Fig.S1 (a) Schematic representation of Ellman's Method. (b) Characteristic graph showing scanning kinetics using Ellman assay. [AChE] = 10 mU/ml, [DTNB] = 100 µM, [ACHI] = 1 mM, Experimental conditions: HEPES buffer = 5 mM, pH 7, T = 25 °C.

3. Michaelis-Menten Curve of enzyme in Buffer

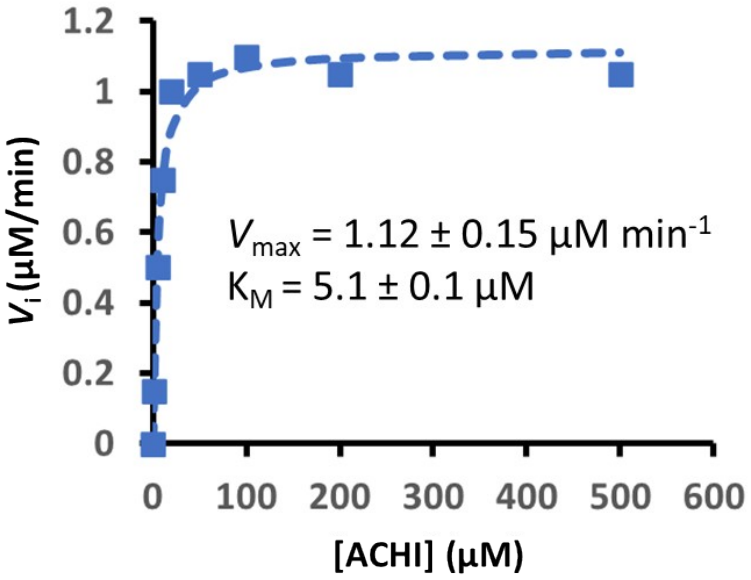


Fig.S2. Representative plot to show V_i of Acetylcholinesterase in buffer ([AChE] = 10mU/ml) catalysis at varying acetylthiocholine iodide concentration (0-500 µM) in buffer (HEPES, pH 7, 5 mM). The lines are the data fit according to a Michaelis–Menten mechanism.

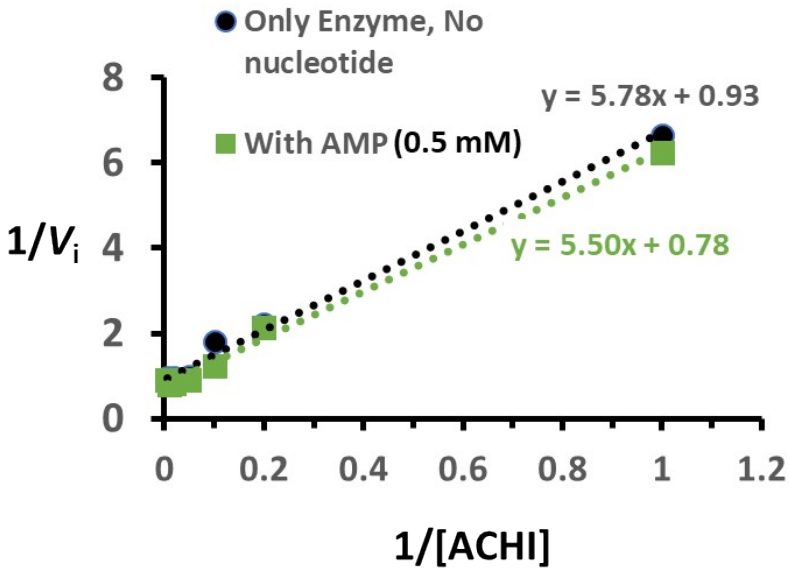


Fig.S3. Lineweaver–Burk plots for the hydrolysis of ACHI in presence of 0.5 mM of AMP [AChE] = 10 mU/ml, [DTNB] = 100 µM, [ACHI] = 1 mM, Experimental conditions: HEPES buffer = 5 mM, pH 7, T = 25 °C.

4. Catalytic activity of Acetylcholinesterase in presence of Paraoxon and long chain alkyl phosphates

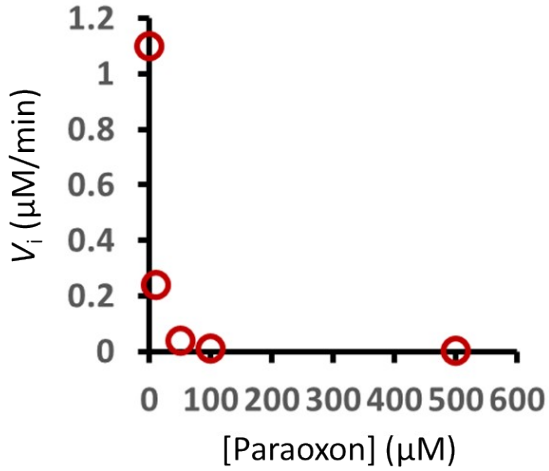


Fig. S4. Initial activity of AChE in presence of different conc. of paraoxon. [AChE] = 10 mU/ml, [DTNB] = 100 µM, [ACHI] = 1 mM, Experimental conditions: HEPES buffer = 5 mM, pH 7, T = 25 °C.

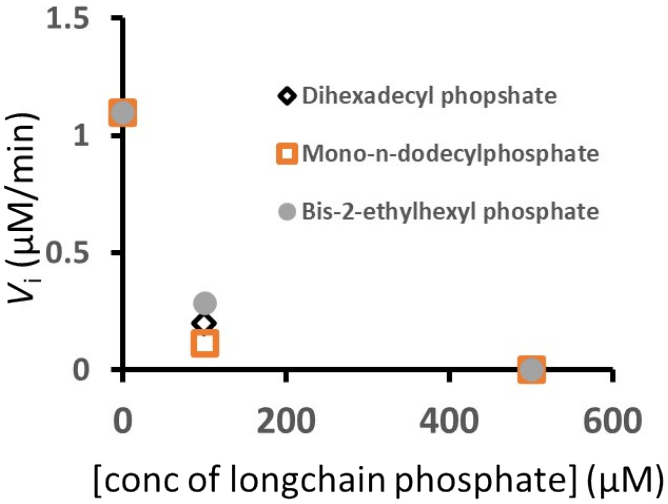
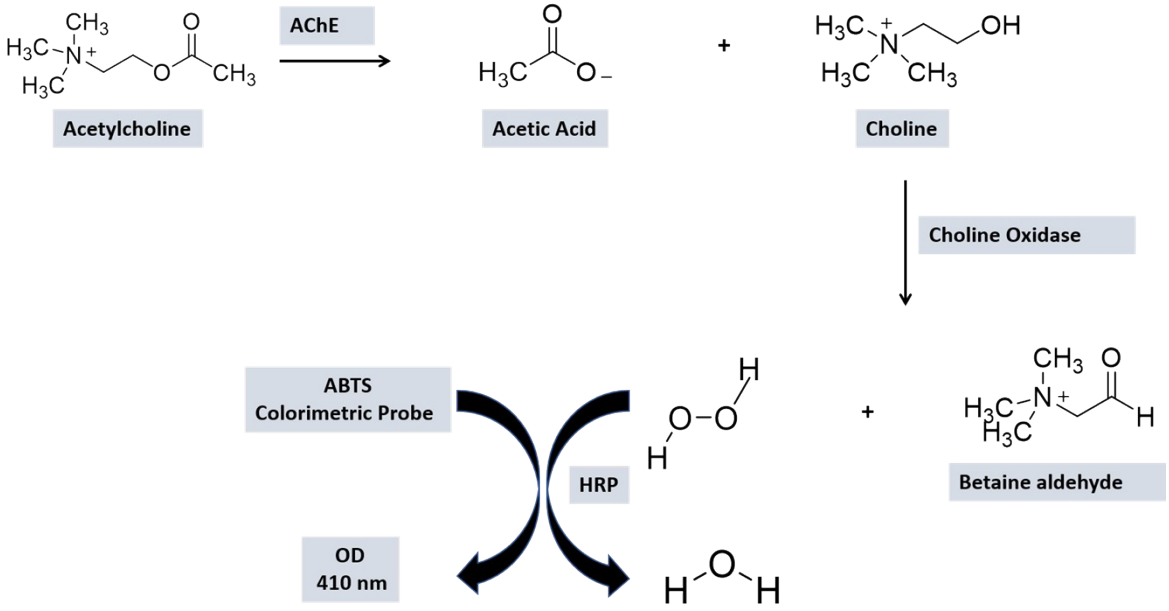


Fig. S5. Representative bar graphs showing V_i of Acetylcholinesterase in buffer ([AChE] = 10mU/ml) with paraoxon and alkyl phosphates. Experimental conditions: HEPES buffer, pH 7, 5 mM, T=25 °C.

5. Choline Oxidase assay with acetylcholine chloride in presence of nucleotides

(a)



(b)

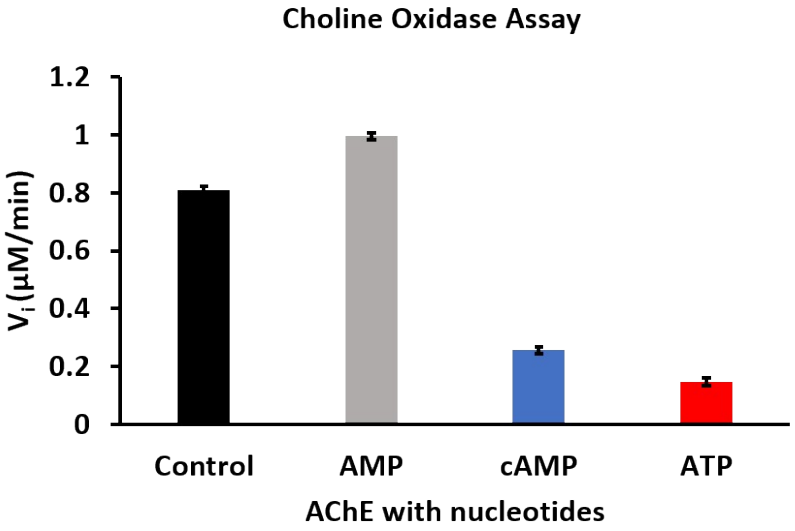


Fig.S6. (a) Pictorial representation of Choline Oxidase Assay. (b) Representative bar graphs showing V_i of Acetylcholinesterase in buffer ([AChE] = 10mU/ml) with different nucleotides having concentration 0.5 mM Experimental conditions: HEPES buffer, pH 7, 5 mM, T=25^{o}C. Choline Oxidase assay was used in order to determine initial rate (Vi).

6. Catalytic activity of Acetylcholinesterase in presence of guanine, thymine, cytosine and uracil based nucleotides:

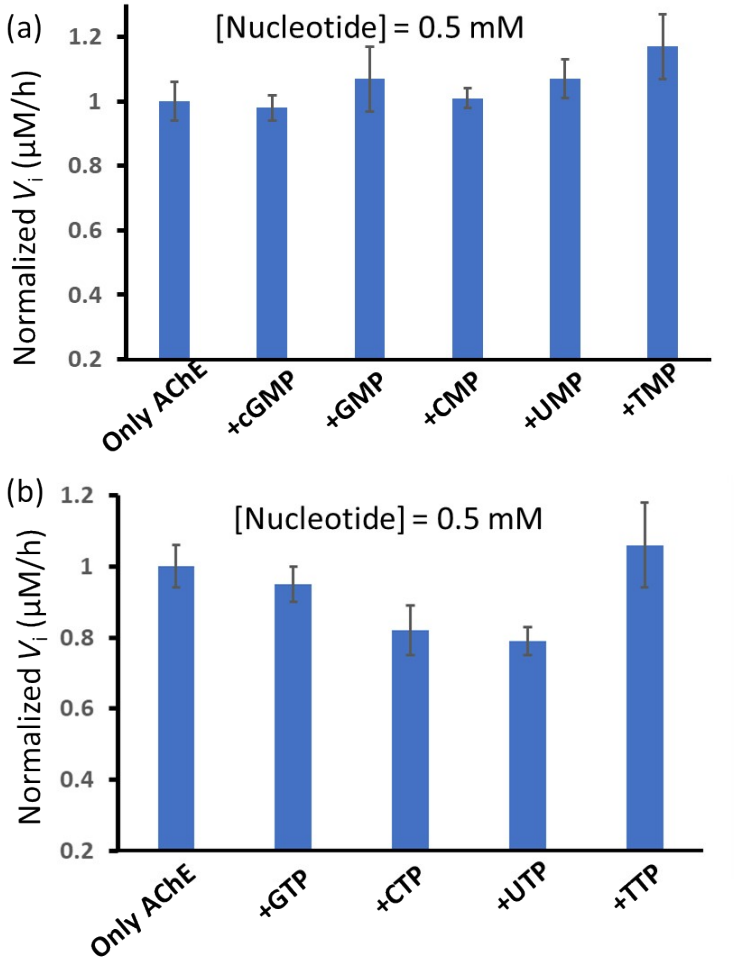


Fig. S7. Comparative plot of normalized initial velocity (V_i) of the enzyme AChE in presence of 500 µM (a) nucleoside monophosphate (GMP, CMP, TMP, UMP) including cyclic guanosine monophosphate (cGMP) and (b) nucleoside triphosphate (GTP, CTP, UTP, TTP) in aqueous buffer (pH = 7, HEPES, 5 mM) in presence of 10 mU/ml AChE and 0.1 mM of substrate, AChI, at T = 25 °C.

This control experiment suggests only adenine-based nucleotides (especially cAMP and ATP) have higher AChE inhibitory ability among all other nucleotides (please compare with Fig. 2a of the main manuscript), suggesting proposed neuromodulatory role of adenine nucleotides (Ref. 10 of the main manuscript).

7. Preparation of Acetylcholine esterase vesicles

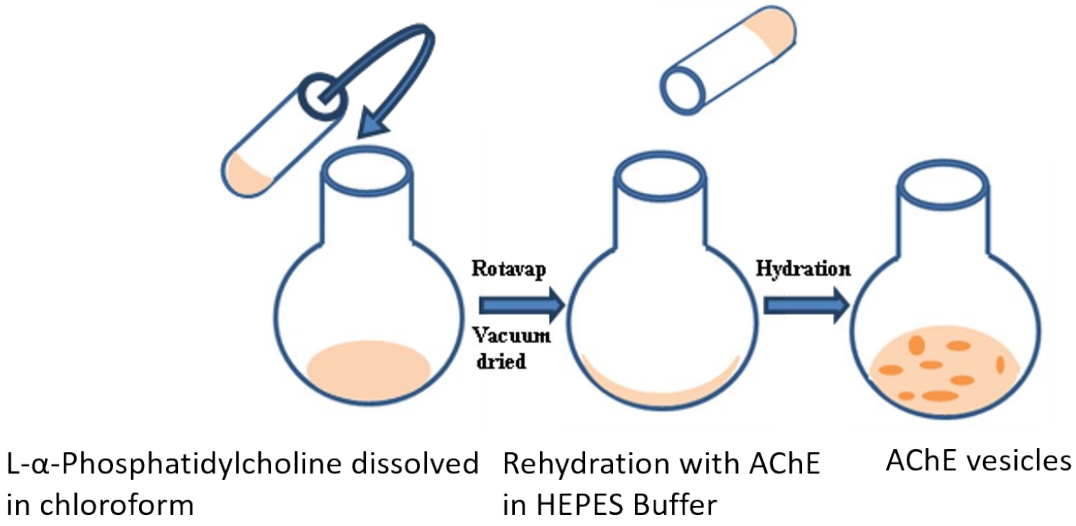


Fig.S8. Schematic representation of preparation of AChE vesicles.

8. Steady State anisotropy of vesicle bound Acetylcholinesterase

Table S1: Anisotropy values of different system containing enzyme.

System	**Anisotropy values**
AChE	0.039
AChE vesicles	0.22
FITC tagged AChE	0.04
FITC tagged AChE vesicles	0.08

Tryptophan steady state anisotropy in case of untagged AChE was measured at excitation wavelength 280 nm and emission at 340 nm with excitation and emission band pass 5 and 7 nm respectively. In case of FITC tagged AChE, steady state anisotropy was measured with the following parameters: λ_{ex}: 480 nm and λ_{em}: 525 nm with excitation and emission bandpass 1.5 and 2 nm respectively.

9. Vesicle bound Acetylcholinesterase activity in presence of nucleotides

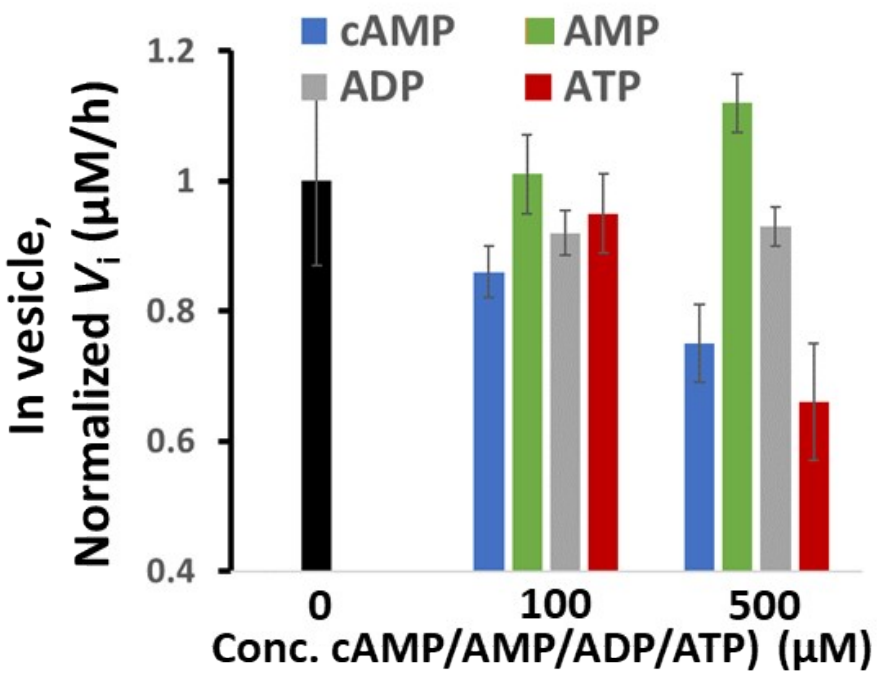


Fig. S9. Representative bar graphs showing V_i of Acetylcholinesterase vesicles in buffer ([AChE] = 10mU/ml) with different nucleotides having concentrations 0.1mM and 0.5 mM. Experimental conditions: HEPES buffer, pH 7, 5 mM, T=25^{o}C. Ellman's assay was used in order to determine initial rate (Vi).

10. Circular Dichroism study of Acetylcholinesterase in presence of nucleotides

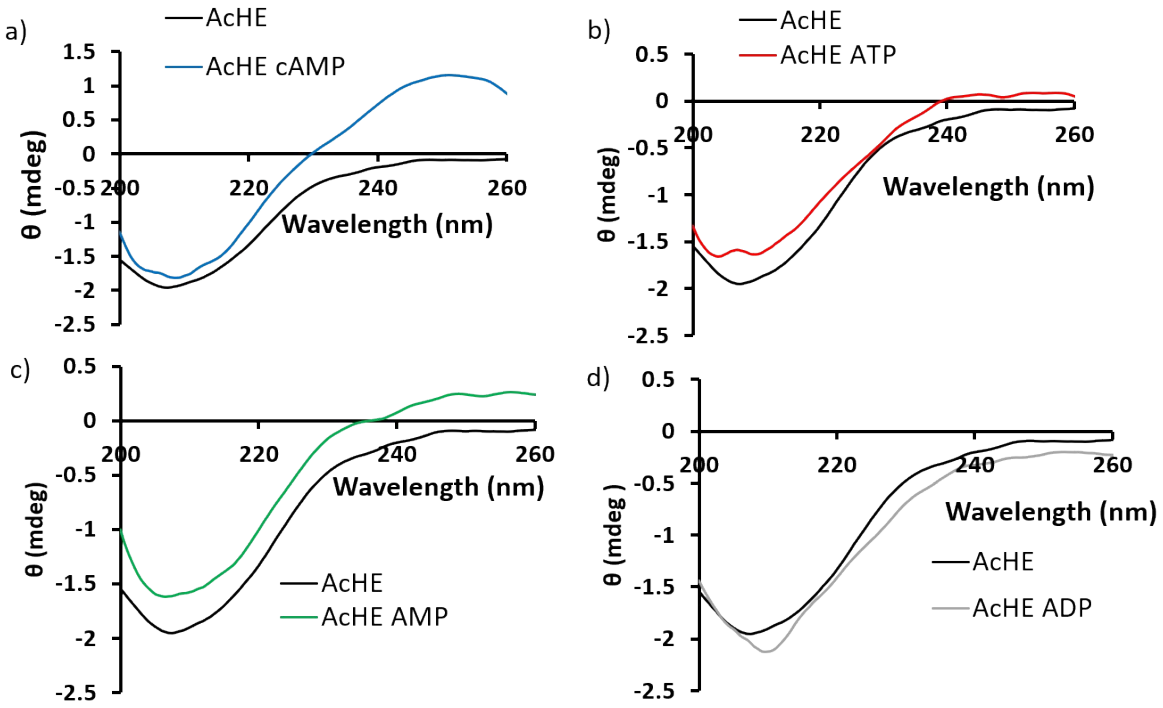


Fig. S10. Circular Dichroism (CD) spectra of only AChE (12 units/ml) along with different nucleotides in Buffer (HEPES, pH = 7). Experimental condition: [AChE] = 12 units/ml, [Nucleotides] = 0.5 mM, CD cuvette pathlength = 1 mm, HEPES buffer (5 mM, pH = 7), T=25 °C.

11. Fluorescence emission spectra of AChE in presence of nucleotides

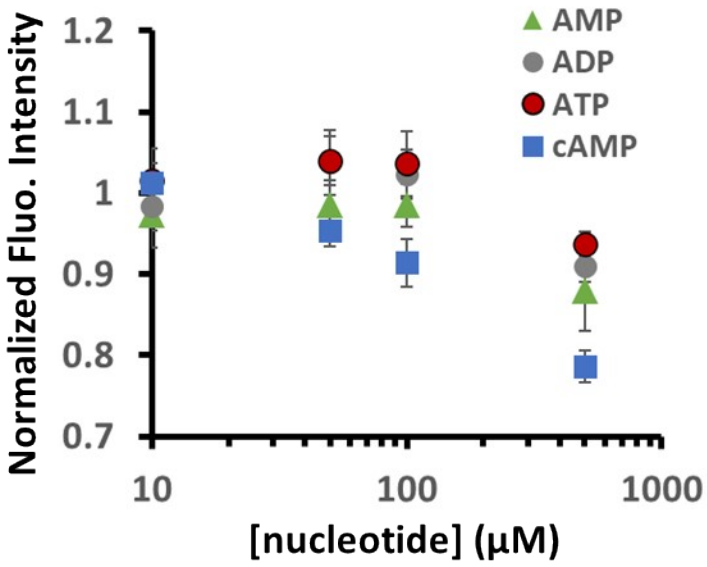


Fig. S11. Normalized fluorescence emission intensity of only AChE (6 units/ml) along with different nucleotides in Buffer (HEPES, pH = 7). Experimental condition: [AChE] = 6 units/ml, [Nucleotides] = 0.5 mM, Excitation wavelength = 290 nm, Slit widths: Excitation/ Emission = 10/10, HEPES buffer (5 mM, pH = 7), T=25 °C.

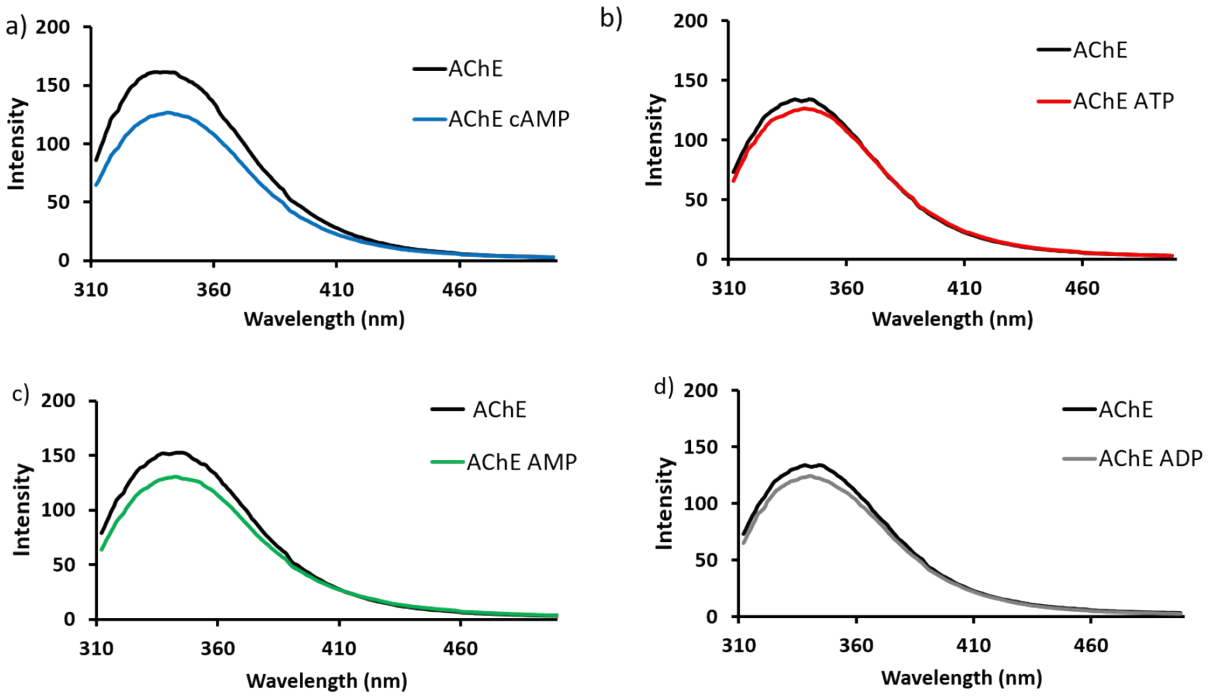
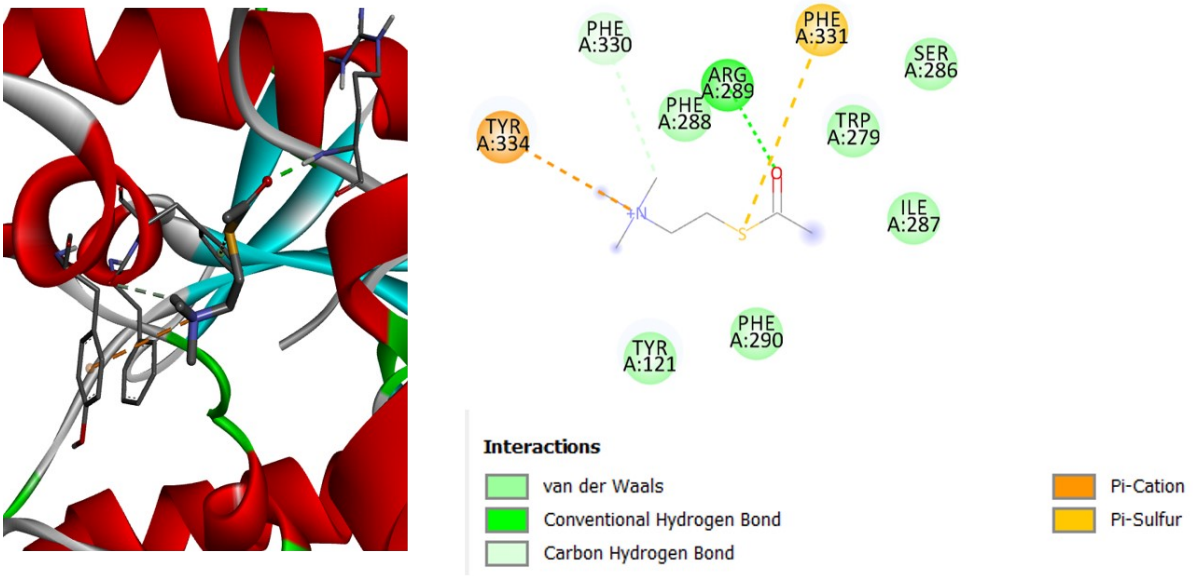


Fig.S12. Fluorescence spectra of only AChE (6 units/ml) along with different nucleotides in Buffer (HEPES, pH = 7). Experimental condition: [AChE] = 6 units/ml, [Nucleotides] = 0.5 mM, Excitation wavelength = 290 nm, Slit widths: Excitation/ Emission = 10/10, HEPES buffer (5 mM, pH = 7), T=25 °C.

12. Molecular Docking Studies

12.1 Docking Interactions



AChE_Acetylthiocholine

Fig. S13. Characteristic picture showing interaction and their types of acetylthiocholine ligand with different amino acid residues of acetylcholinesterase. PDB Id: 2ACE

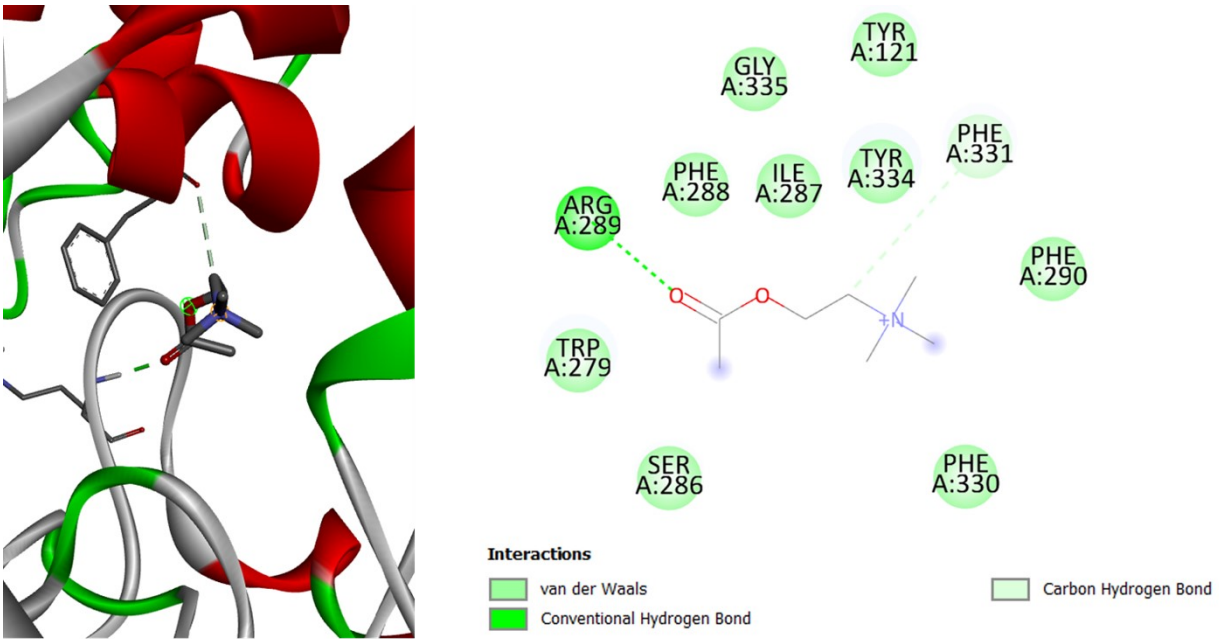
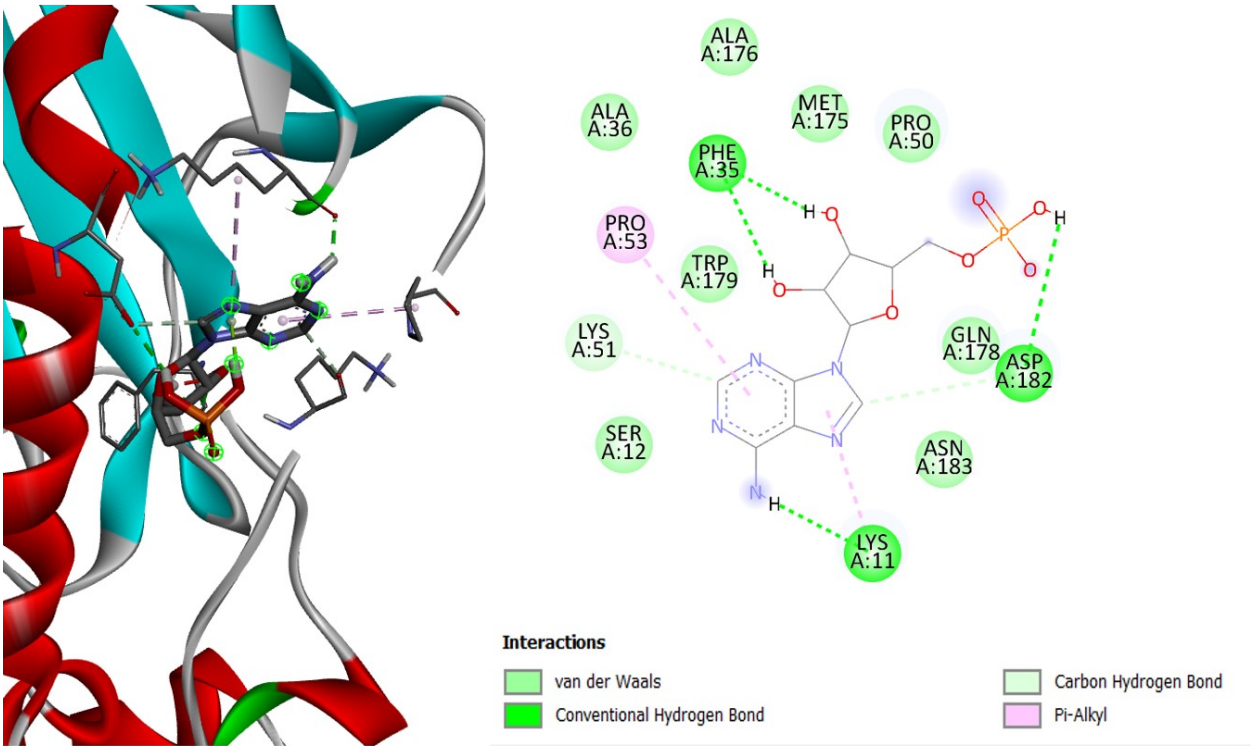
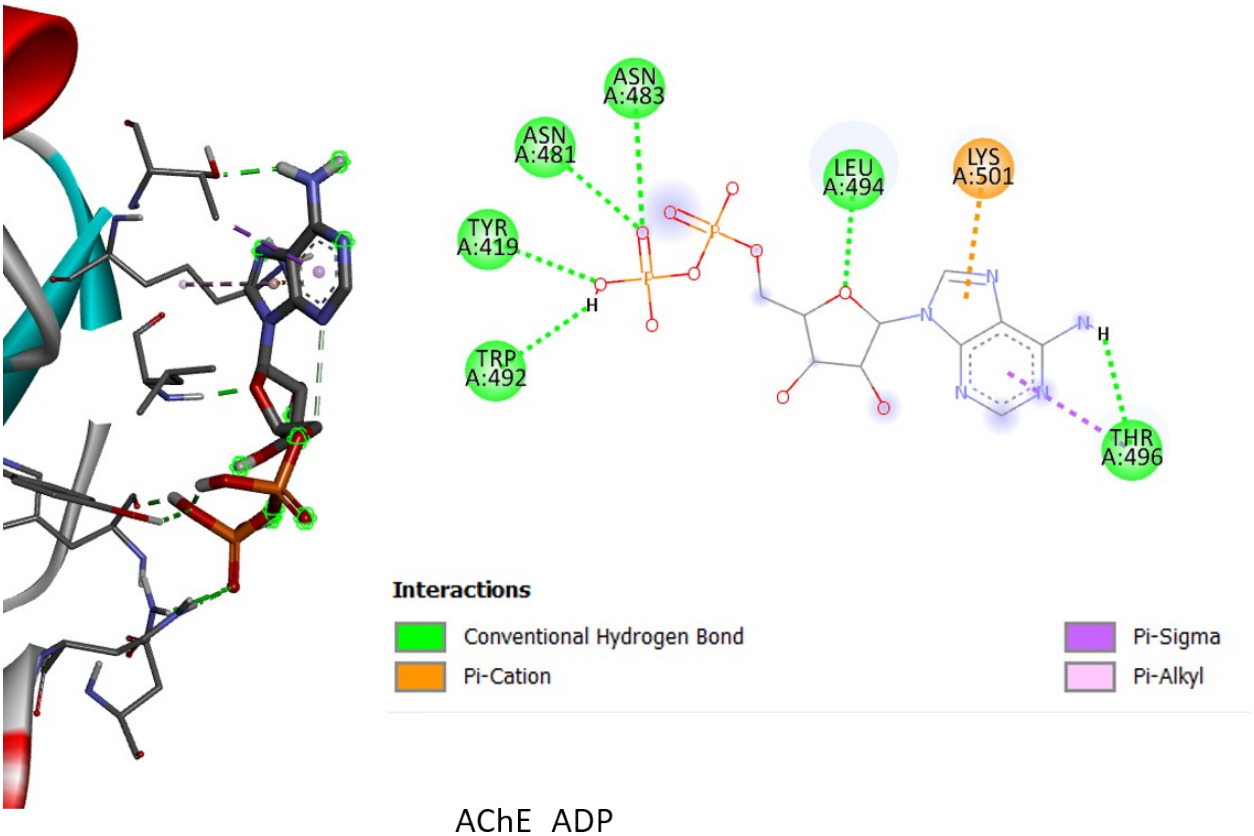


Fig. S14 Characteristic picture showing interaction and their types of acetylcholine ligand with different amino acid residues of acetylcholinesterase. PDB Id: 2ACE



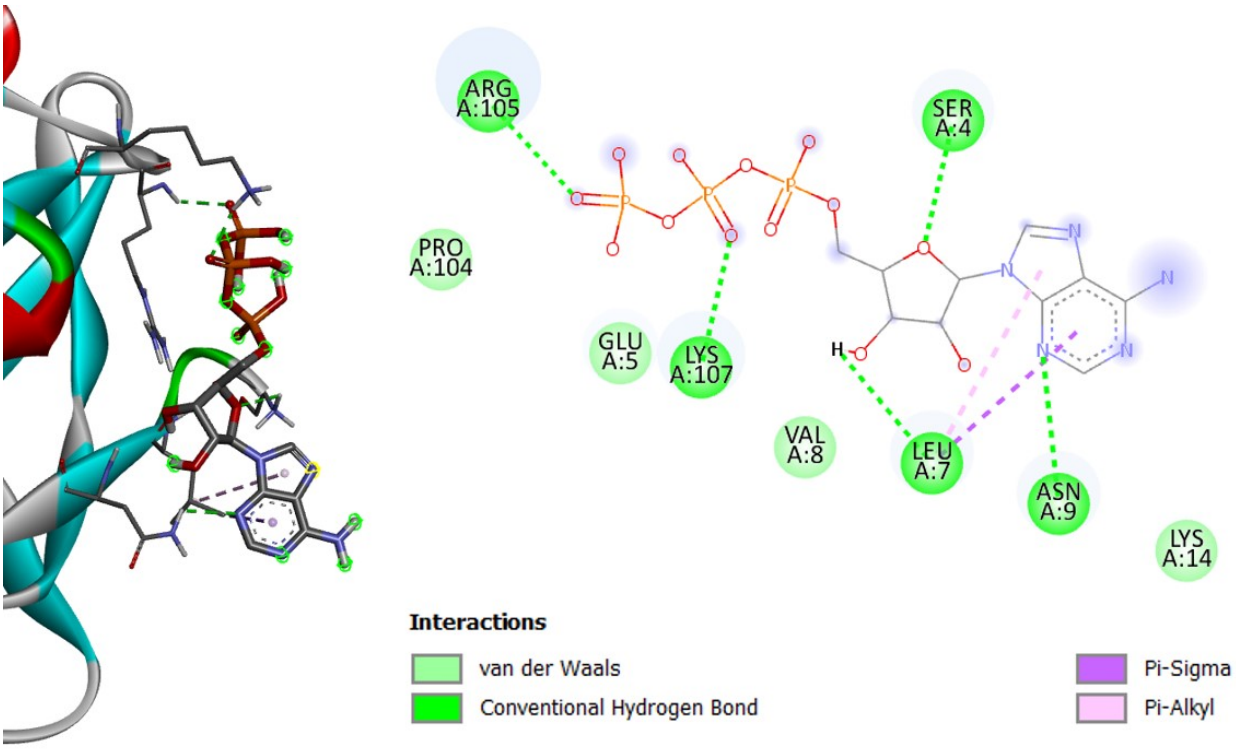
AChE_AMP

Fig. S15. Characteristic picture showing interaction and their types with adenosine monophosphate (AMP) ligand with different amino acid residues of acetylcholinesterase. PDB Id: 2ACE



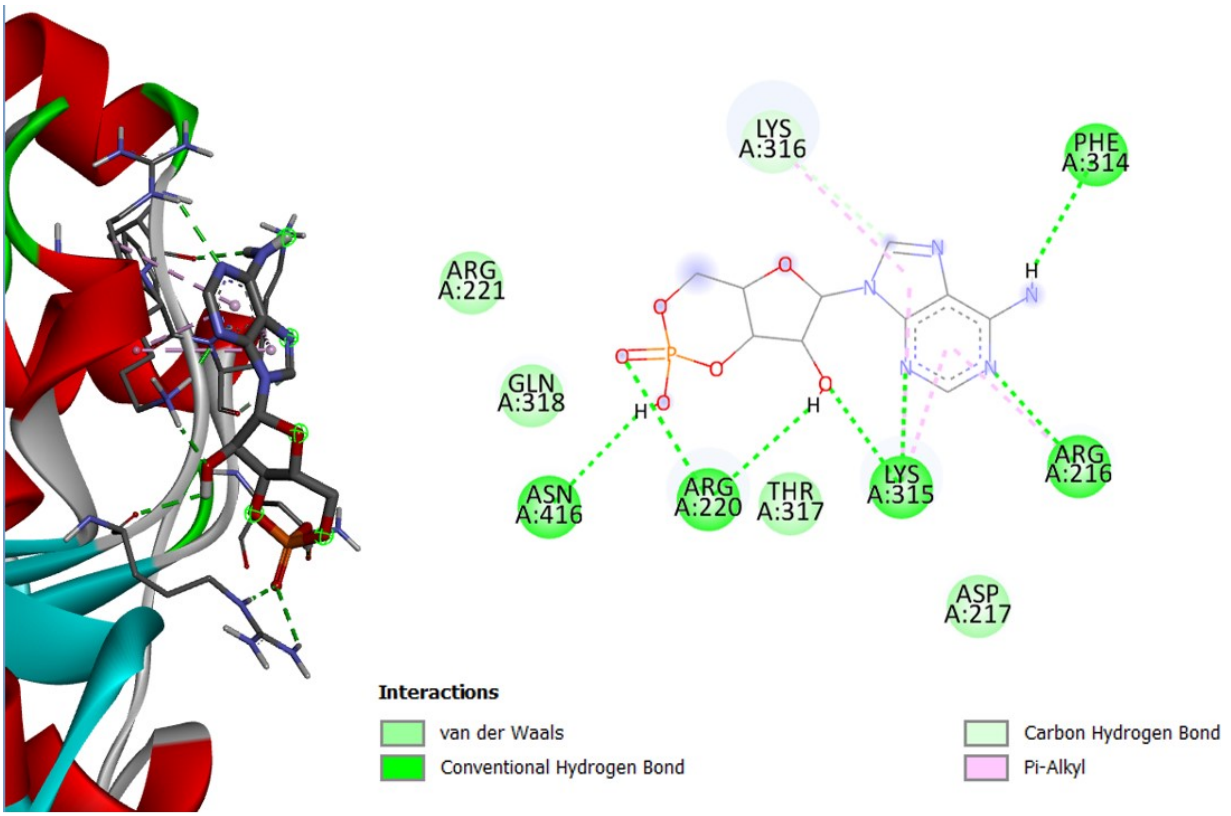
AChE_ADP

Fig. S16 Characteristic picture showing interaction and their types with adenosine diphosphate (ADP) ligand with different amino acid residues of acetylcholinesterase. PDB Id: 2ACE



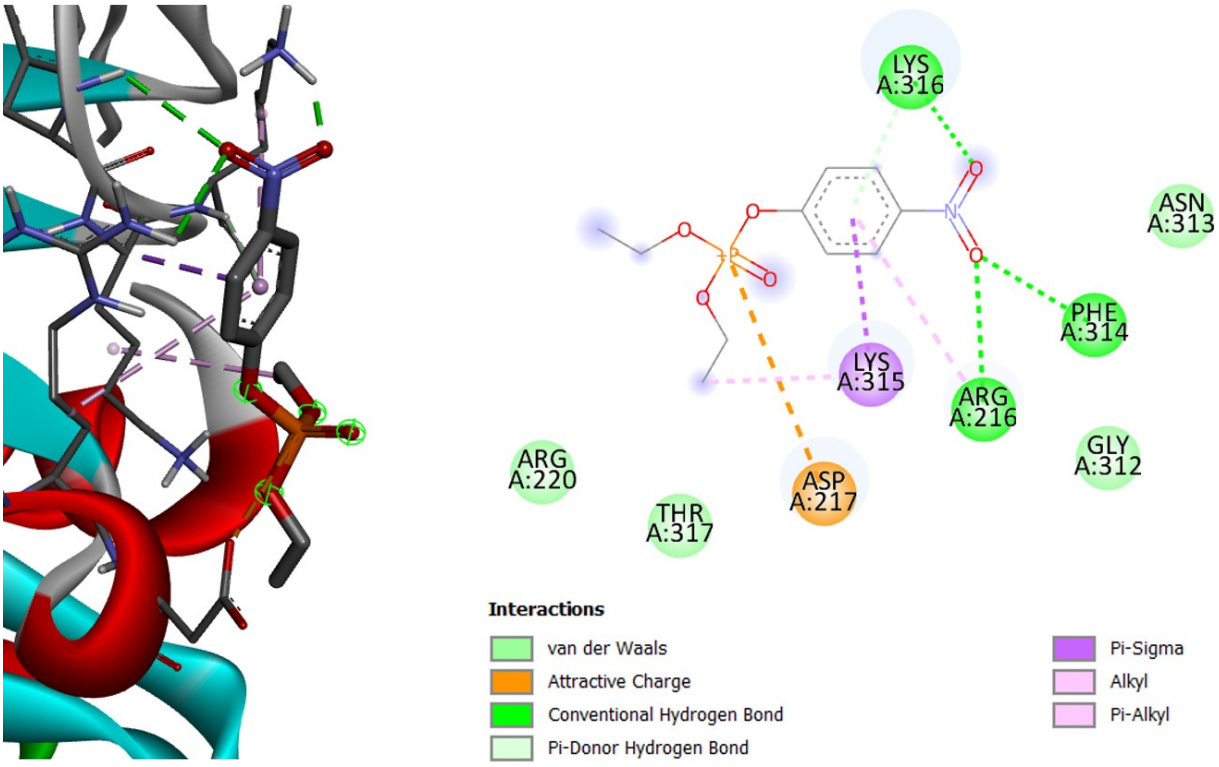
AChE_ATP

Fig. S17 Characteristic picture showing interaction and their types with adenosine triphosphate (ATP) ligand with different amino acid residues of acetylcholinesterase. PDB Id: 2ACE



AChE_cAMP

Fig. S18. Characteristic picture showing interaction and their types with cyclic adenosine monophosphate (cAMP) ligand with different amino acid residues of acetylcholinesterase. PDB Id: 2ACE



AChE_Paraoxon

Fig. S19. Characteristic picture showing interaction and their types with Paraoxon ligand with different amino acid residues of acetylcholinesterase. PDB Id: 2ACE

13. Fabrication of multilayer enzyme-powered micropump and tracking fluid flow.

The multilayer enzyme-powered micropump was fabricated by immobilizing enzyme AChE and plasma on glass substrate via LbL deposition technique. To construct a circular multilayer-enzyme pattern on the glass slide, a thin layer of PDMS sheet with a small circular aperture in the centre of 4.6mm diameter was first made and pasted on the glass slide in such a manner that the circular opening remained in the middle of the slide. The subsequent LbL deposition was achieved for desired number of enzyme layers on the glass substrate. AChE (3U/ml in Tris buffer) and plasma were used as negatively charged polyelectrolytes and PEI (0.1 wt%) was used as positively charged polyelectrolyte for the construction of polymer-enzyme multilayer films. The PDMS sheet was peeled off after producing the desired multilayer coating, and an enzyme pattern with a diameter of 4.6mm was produced in the centre of the glass slide, leaving the covered region uncoated. The enzyme functionalized surface was extensively washed with buffer to remove any unbound or loosely bound enzyme and dried with nitrogen. Then, on the glass slide, an imaging chamber was created by covering the pattern with an airtight imaging chamber (l= 1 cm, b= 1 cm, h = 1.8 mm) and injecting a corresponding buffered substrate solution along with 5 µm diameter neutral charged non-interacting polystyrene particles (tracer particles) into the chamber. The fluid flow was then tracked using an optical microscope at a distance of 480 µm (bottom layer) above the glass slide's surface, and movies were recorded for subsequent analysis using Tracker software. The fluid pumping velocity was measured based on the analysis of the motion of the tracer particles imaged with digital cameras and is the standard measurement technique in fluid dynamics research. In each experiment, ~25 tracer particles were monitored during a 25-second time period using Tracker software to determine fluid-pumping velocity.

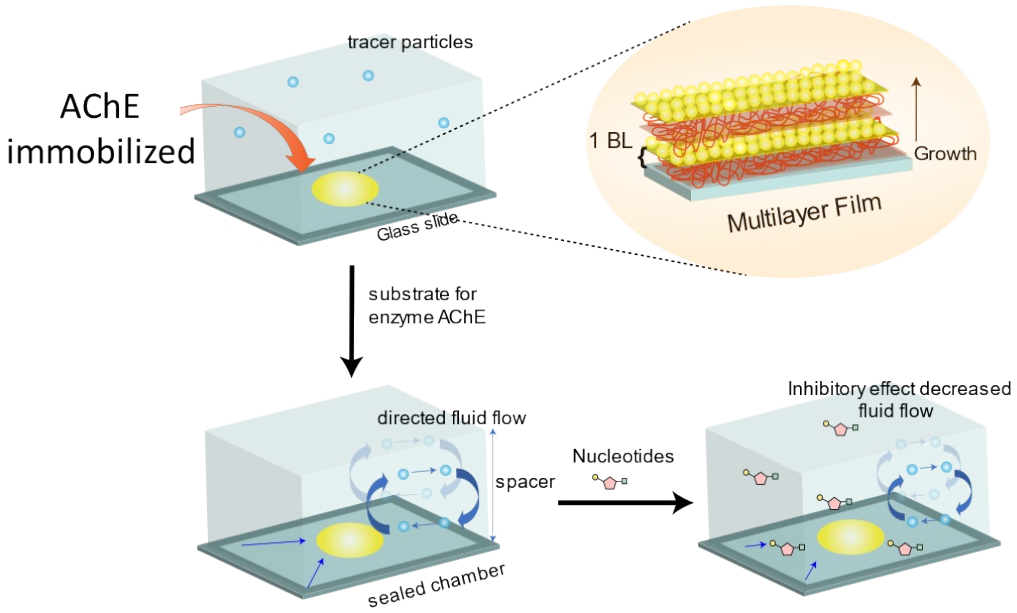


Fig. S20. Schematic representation of the experimental set up.

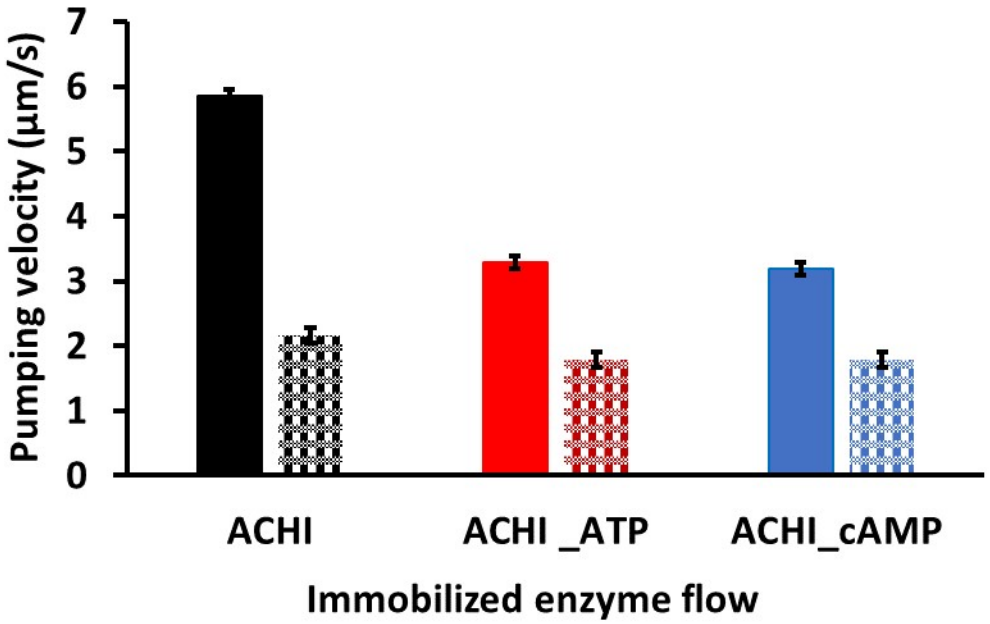


Fig. S21. Obtained flow velocity in presence of adenosine-based nucleotides with immobilized AChE. [ACHI] = 0.1 mM.

14. Supplementary Videos

14.1 Supplementary video 1 (SV1): Molecular docking video of acetylcholinesterase with acetylthiocholine iodide (AChE_ACHI).

14.2 Supplementary video 2 (SV2): Molecular docking video of acetylcholinesterase with adenosine triphosphate (AChE_ATP).

14.3 Supplementary video 3 (SV3): Molecular docking video of acetylcholinesterase with cyclic adenosine monophosphate (AChE_cAMP).

14.4 Supplementary video 4 (SV4): Molecular docking video of acetylcholinesterase with paraoxon (AChE_Paraoxon).

14.5 Supplementary video 5 (SV5): Flow of 1 mM substrate acetylthiocholine iodide (ACHI) on Plasma bilayer film (4x speed).

14.6 Supplementary video 6 (SV6): Flow of 1 mM substrate acetylthiocholine iodide (ACHI) in presence of 0.5 mM ATP on Plasma bilayer film (4x speed).

14.7 Supplementary video 7 (SV7): Flow of 1 mM substrate acetylthiocholine iodide (ACHI) in presence of 0.5 mM cAMP on Plasma bilayer film (4x speed).

14.8 Supplementary video 8 (SV8): Flow of 1 mM substrate acetylthiocholine iodide (ACHI) in presence of 0.5 mM paraoxon on Plasma bilayer film (4x speed).

15. References.

1. A. Micsonai, F. Wien, E. Bulyáki, J. kun, E. Moussong, H. Y. Lee, Y. Goto, M. Réfrégiers and J. Kardos, *Nucleic Acids Research* 2018, **46**, W315–W322.

2. A. K. Gill, R. Varshney, M. Alam, C. Agashe and D. Patra , *ACS Appl. Bio Mater.* 2021, **4**, 6203–6208.

3. P. Walde and S. Ichikawa, *Biomol. Eng.* 2001, **18**, 143–177.

4. A. Deshwal, H. Chitra, M. Maity, S. K. Pal and S. Maiti, *Chem. Commun.* 2020, **56** 10698–10701.

5. E.Reiner and V. Simeon-Rudolf , *Toxicology of Organophosphate & Carbamate Compounds*; *Elsevier*, 2006; pp 199–208.