Supporting information

One-pot Bottom-up Synthesis of 2D Graphene Derivative: Application in Biomolecular Recognition and Nanozyme Activity

*Subrata Pandit and Mrinmoy De**

Department of Organic Chemistry, Indian Institute of Science, Bangalore-560012, India

Email: md@iisc.ac.in

Materials. 2,4,6-tribromo-3-hydroxybenzoic acid (TBHBA), n-Butyllithium (n-BuLi), Ferrous chloride ($FeCl_2$), Triethylamine($C_6H_{15}N$), α-Chymotrypsin (α-ChT) and *N*-Succinyl *L*-Phenylalanine *p*-Nitroanilide (SPNA, 99 %) were purchased from Sigma Aldrich. Sodium Phosphate Dibasic Heptahydrate (Na_2HPO_4 $7H_2O$, 95%) and Sodium Phosphate Monobasic (NaH_2PO_4, 99%), Dimethyl Sulphoxide (DMSO, 99.5%), Ethanol (EtOH, 99.9%) and solvent Tetrahydrofuran (THF) were purchased from SD fine chemicals Ltd. 5 mM sodium phosphate buffer solution at pH 7.4 was prepared and used for respective studies.

Synthesis of 2D nanosheets: We have synthesized 2D carbon-based nanomaterial using 2, 4, 6-tribromo-3-hydroxybenzoic acid (TBHBA) (0.5 mmol) as the starting material in the presence of 1.6 M n-butyl lithium (n-BuLi) (1.23 ml, excess) and ferrous (II) chloride (20 mol %) as a catalyst along with triethylamine (0.7 mL) as an additive in 10 ml dry tetrahydrofuran (THF) solvent at -78 ^oC for 3 days. We had observed that during the progress of the reaction, the colour of the reaction mixture changed from pale yellow to dark brown. This suggests that self-polymerization has taken place. The reaction mixture was quenched by few drops of 1N HCl solution, followed by organic solvent was evaporated. The obtained black residue was redispersed in distilled water and centrifuged at 4000 rpm to remove the insoluble residues. Finally, the prepared material was purified through dialysis in 5 mM sodium phosphate buffer.

After the purification the synthesized material was characterized by various microscopic and spectroscopic techniques.

Activity Assay. All the activity assays were performed using 5 mM sodium phosphate buffer, pH 7.4 at 25 ^{o}C. We have prepared α-ChT solution 3.2 μM (12.8 μM stock solution of α-ChT) which was incubated with different concentrations of the synthesized 2D nanosheet ranging from 0 to 45 μg/mL (80 μg/mL stock solution). The synthesized 2D nanosheet with α-ChT (180 μL) mixture was allowed for 30 mins to form a complex and then 20 μL of chromogenic substrate SPNA was added, which was prepared by 10% DMSO in EtOH solution. The enzymatic activity was monitored by measuring the absorbance of the hydrolysis product *p*-nitro aniline (PNA) at a particular wavelength of 405 nm with 15 min interval for up to 3 h using micro plate reader Instrument (VARIOSKAN FLASH).

Kinetics Study. We have analyzed the mode of inhibition of α-ChT by the synthesized 2D nanosheet through kinetics assay. The experiment was performed in 5 mM Sodium Phosphate buffer solution at pH 7.4. At first, we fixed the material concentration in all the wells and varied the substrate concentration from 0 to 80 μM. Similarly, we fixed substrate concentration in all the well and varied the inhibitor concentration from 0 to 14 μg/mL and α-ChT concentration was always fixed at 3.2μM.

Circular Dichroism (CD) Study. We have performed the Circular Dichroism (CD) study of α-ChT to analyze the effect of synthesized material on secondary structure. We measured CD of the complex between the synthesized 2D nanosheet with α-ChT and only native α-ChT at different time intervals in sodium phosphate buffer 5 mM at pH 7.4 solution. The spectra were recorded for wavelength ranging from 240 nm to 190 nm using JASCO Circular Dichroism Spectrophotometer J-810, in quartz cuvettes of 2 mm path length at 25 ^{o}C. Three rounds of assay have been performed and the average value is reported here.

Fluorescence Study. We have also performed the fluorescence study of native α-ChT and as well as that of the complex between the synthesized 2D nanosheet and α-ChT at different time interval in 5 mM sodium phosphate buffer at pH 7.4. The emission wavelength of α-ChT as well as the complex have been recorded by applying the excitation wavelength 295 nm on Varian Fluorescence Spectrophotometer Instrument.

Zeta Potential measurement of 2D Nanosheet. Zeta potential (ζ) experiment was performed in 5 mM phosphate buffer at pH 7.4 at 25 ^{o}C. The sample (synthesized 2D nanosheet) was measured by using MALVERN Zetasizer Nano ZS Instrument and three rounds of measurements were performed and the average value was reported.

Nanozyme Activity Assay. Enzyme-like activity of bottom-up approach 2D nanosheets has been performed using Sodium Phosphate buffer solution (5 mM, pH 7.4) at 25 °C. In study, the final concentration of prepared materials was used ranging from 0 - 75 μg/mL (stock solution conc. 0.15 mg/mL). Also, we have added 20 μL solution of each H_2O_2 and NADH to reach the final concentration 0.5 mM for both the substrate. After catalyzed the reaction we have measured the absorbance of NADH at 340 nm for 5 min intervals. Similarly, we have also performed Dopamine activity assay, after catalyzed the reaction we have measured the absorbance of the oxidized product at 480 nm for 5 min intervals.

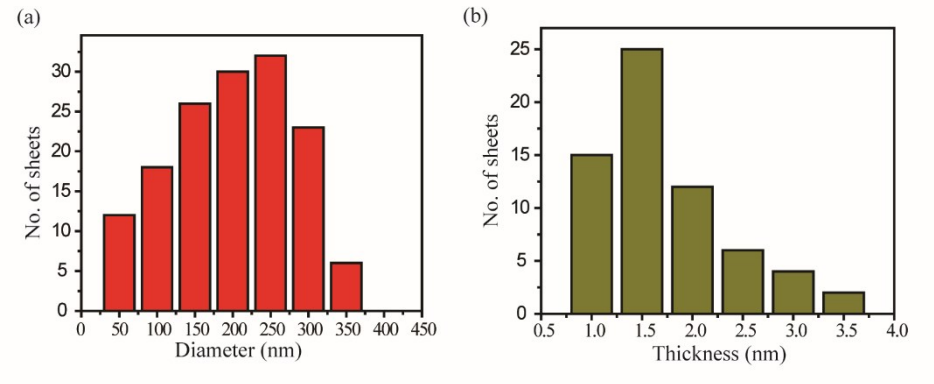
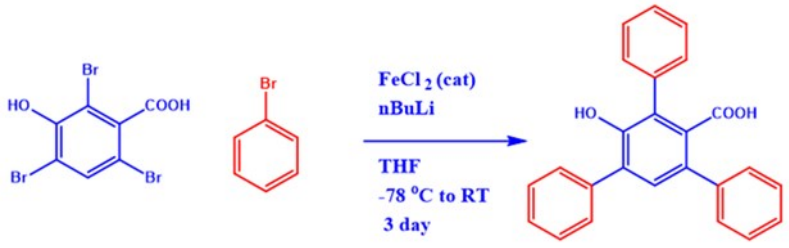


Figure S1. Size distribution plot (a) and thickness profile diagram (b) of synthesized 2D nanosheets.



Reaction scheme of Monomer:

Figure S2. ^{1}H Spectra of 2,4,6-triphenyl -3-hydroxybenzoic acid in DMSO-D6 Solvent. ^{1}H-NMR of compound Monomer. ^{1}H-NMR (400 MHz, DMSO-d6): δ : 13.98 (s, 1H, -COOH-), 10.58 (s, 1H, -OH), 7.98 (s, 1H, CH) 7.72-6.63(m, 15H, -CH-).

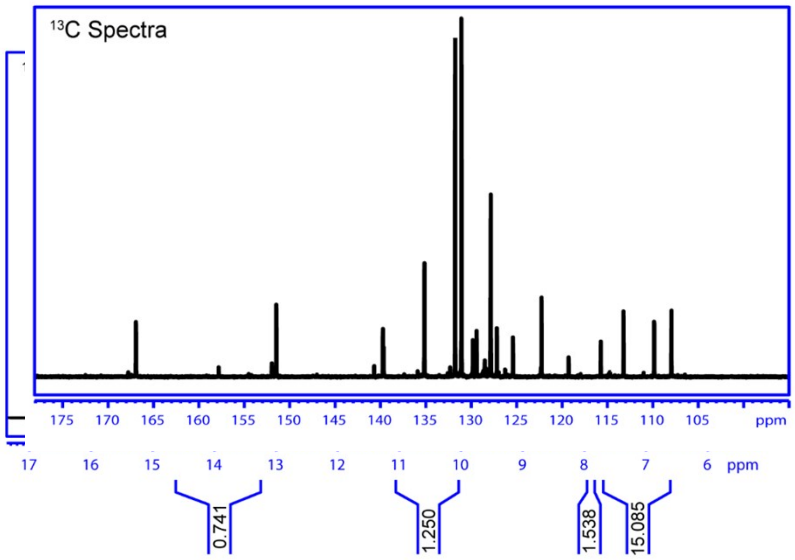
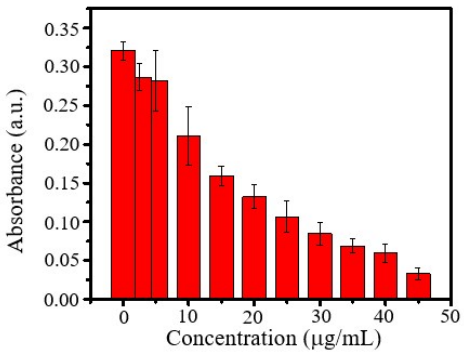


Figure S3.^{13}C NMR Spectra of 2,4,6-triphenyl -3-hydroxybenzoic acid in DMSO-D6 Solvent. ^{13}C-NMR of compound Monomer. ^{13}C-NMR (400 MHz, DMSO-d6): δ: 167.2 (COOH), 151.6 (C-OH), 139.8, 135.0, 131.9, 131.0, 129.8, 129.3, 127.9, 127.2, 125.5, 122.3, 119.9, 115.6, 113.1, 109.9 and 107.8 (C-H).



Enzymatic activity of α-Chymotrypsin:

Figure S4. The study of enzymatic activity (α-ChT) in the presence of synthesized 2D-nanosheets in 5 mM Phosphate buffer at pH 7.4.

Figure S5. . Enzymatic activity of α-ChT in the presence bottom-up approach 2D nanosheets at (a), percentage of inhibition at different pH (b) and Circular Dichroism study of α-ChT (c) different pH ranging from 4 to 10.

Figure S6. Enzymatic activity of α-ChT in the presence bottom-up approach 2D nanosheets in serum (2-16%) at pH 7.4.

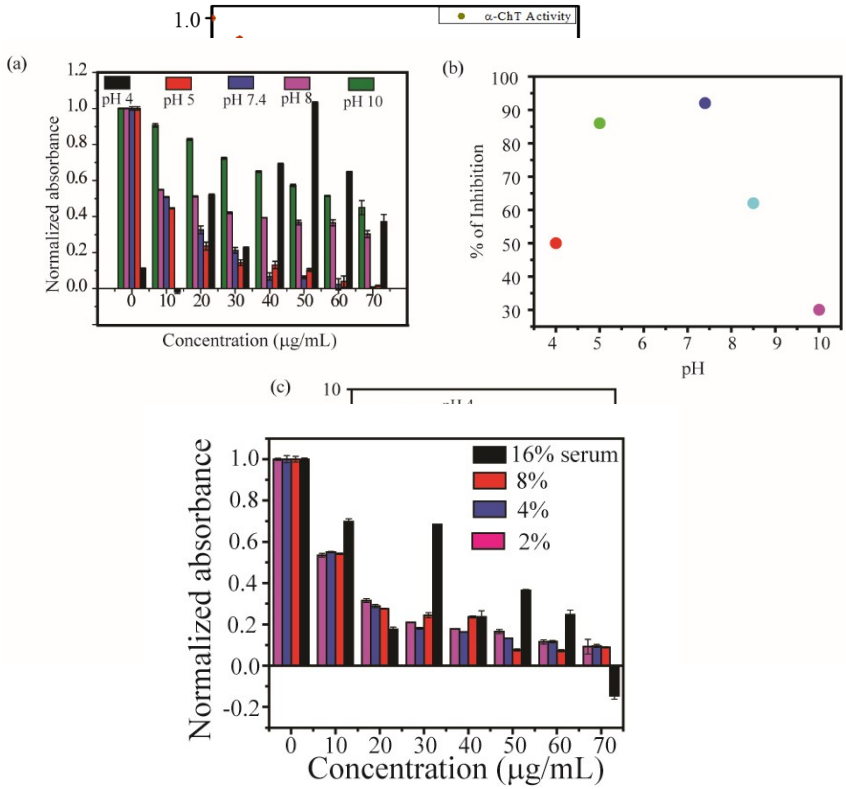


Figure S7. The study of enzymatic activity (α-ChT) with time in the presence of synthesized 2D-nanosheets in 5 mM Phosphate buffer at pH 7.4.

Table S1: Table for percentage of inhibition with different materials at respective concentration.

Table S2: Secondary structure determination using Fluorescence Spectroscopy technique in 5 mM sodium phosphate buffer solution at pH 7.4.

Name of the samples	(%) of denaturation 0h	(%) of denaturation 2h	(%) of denaturation 4h	(%) of denaturation 8h
2D nanosheet vs. α-ChT	3.5	6	8	12
Only α-ChT	2	3.5	6	8

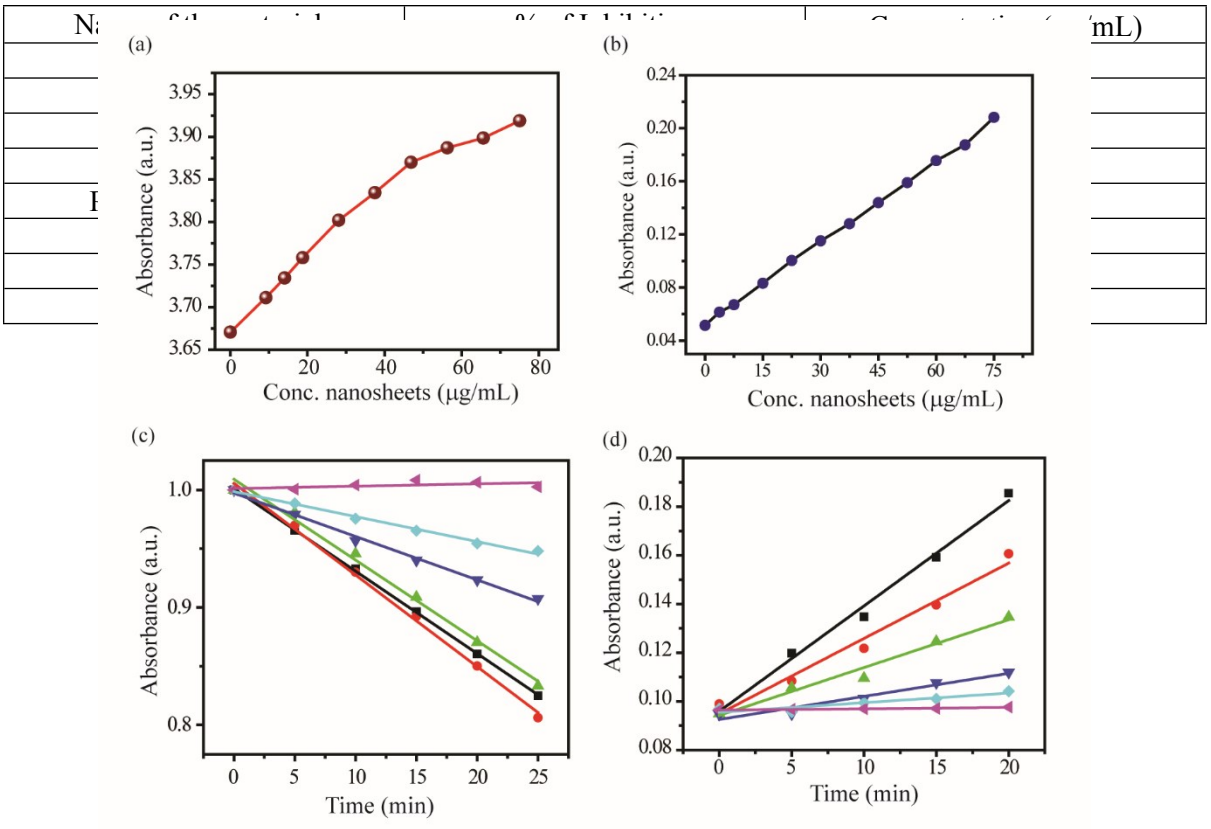


Figure S8. Nanozyme activity assay of NADH (a, c) and Dopamine (b, d) with increasing concentration of synthesized 2D-nanosheets in 5 mM Phosphate buffer at pH 7.4