Electronic Supplementary Material (ESI) for Analyst.

Correlating enzyme density, conformation and activity on nanoparticle surface for high functional bio-nanocomposite

Bedabrata Saha,[a] *Jiban Saikia,*[b] *Gopal Das*[a,b*]

[a]*Centre for the Environment, Indian Institute of Technology Guwahati, Assam-781039, India*

[b]*Department of Chemistry, Indian Institute of Technology Guwahati, Assam-781039, India*

Supplementary Information

Experimental Section:

Characterization of CuS nanoparticles

Synthesized CuS nanoparticles were characterized in transmission electron micrograph (TEM, JEOL 2100 UHR-TEM). A small volume (5 μL) of CuS nanoparticle dispersion solution was drop-cast on carbon-coated copper grids and subsequently air-dried before TEM analysis. Selective area electron diffraction (SAED) of CuS nanoparticles were also determined from the TEM analysis. Specific surface area and pore volume of CuS nanoparticles were measured using a *Beckman Coulter SA3100* surface area analyzer, by measuring N_2 adsorption and adopting the well-known BET procedure.[1] Energy-dispersive X-ray analysis of CuS nanoparticles was performed for chemical characterization, using a *LEO-1430 VP* scanning electron microscope. FT-IR spectra of CuS nanoparticles were recorded from 4000 to 450 cm^{-1} range (at 4 cm^{-1} resolution with 10 scans) in a *Perkin-Elmer Spectrum One* FT-IR spectrometer. Thermogravimetric analysis (TGA) and differential scanning calorimetric (DSC) measurement of CuS nanoparticles was performed by using an SDTA 851e TGA thermal analyzer (*Mettler Toledo*) with a heating rate of 2 °C/min in N_2 atmosphere. Powder X-ray diffraction (PXRD) data was recorded with Seifert powder X-ray diffractometer (XRD 3003TT) with Cu K_α source (λ =1.54 Å) on a glass surface of an air-dried CuS nanoparticle sample. The average crystallite size of the CuS nanoparticles was estimated using the Debye−Scherrer equation.[2]

Adsorption of trypsin

Batch adsorption experiment was performed in a 2 mL vial by adding different concentration of CuS nanoparticles to trypsin solution, and by continuously stirring in a temperature controlled end-over rotor. All the adsorption experiments were performed at pH 8.0 (with 10 mM Tris-HCl buffer) as trypsin possess its optimum activity at this pH.[3] The enzyme bound nanoparticles were separated from unbound enzymes in solutions by centrifugation at 15000 rpm for 15 min, and the supernatant solution was estimated for unbound enzyme concentration. The initial and final (from the supernatant) enzyme concentration was determined by well-known Bradford assay procedure by measuring optical density at 595 nm, and as well as by directly measuring the protein absorbance at 280 nm. The amount of adsorbed enzyme (Γ_{trp}, mg m^{-2}) on nanoparticle surface was calculated from initial (C_0, mg mL^{-1}) and final (C_e, mg mL^{-1}) enzyme concentration and specific surface area (m^2 g^{-1}) of CuS nanoparticles.

Adsorption kinetics of trypsin was studied by withdrawing the samples from the reaction mixture at regular time interval, and analyzing the supernatant trypsin concentration as a function of time. A fix trypsin concentration of 0.5 mg mL^{-1} and CuS nanoparticles of 1.0 mg mL^{-1} have been used for kinetic

studies. For different enzyme surface density, an increased concentration of trypsin (0.1 – 1.0 mg mL^{-1}) was added to a fix amount of CuS nanoparticles (1 mg mL^{-1}), and incubated for 6 h at room temperature. Our preliminary studies showed this period of time is sufficient to ensure steady state for the above mentioned concentration range. Trypsin interaction with CuS nanoparticles were also studied at a function of different available surface area, by using different concentration ratio of trypsin and CuS nanoparticles.

The approximate available surface area could be calculated from the nanoparticle concentration (mg mL^{-1}) and its specific surface area (m^2 g^{-1}). However, this value can be deviated to some extent due to the variation in the size distribution and partial aggregation possibilities. Further we have also analyzed the adsorption capacity, thermodynamic parameters, conformation and activity of trypsin adsorption in a temperature range from 25 ^oC to 50 ^oC, by incubating 0.2 mg mL^{-1} trypsin solution with 0.1 mg mL^{-1} CuS nanoparticles. The temperature of the system was controlled in an indigenous made closed circulating water bath. First, the trypsin with CuS nanoparticles were incubated at a defined temperature for adsorption, thereafter the supernatant was analyzed for adsorption capacity, and the enzyme coated nanoparticles were then placed back (resuspending in enzyme free buffer and in presence of substrate) at the same temperature for activity analysis. The conformational studies of trypsin-CuS interaction at different temperature were performed by measuring the steady state fluorescence emission intensity, in a temperature controlled thermostatted cell holder within fluorescence spectrophotometer.

Conformational analysis of trypsin interaction with CuS nanoparticles

Fluorescence spectra for tertiary structure. The tertiary conformation of native trypsin in solution and in adsorbed state on nanoparticle surface was analyzed using tryptophan fluorescence emission technique in a steady-state FSP920 spectrofluorimeter (*Edinburgh Instrument*). The excitation wavelength at 290 nm was chosen because almost all the fluorescence emission signal excited at this wavelength is derived from tryptophan. Typically, the fluorescence spectra of trypsin interaction with CuS nanoparticles was measured as a function of: time, enzyme surface density, temperature, trypsin concentration and nanoparticle concentration. In general a lower concentration of CuS nanoparticles were used for all fluorescence spectroscopic measurements, which is specified later for each case during the discussion section, to minimize optical scattering by the nanoparticles. Steady-state anisotropy measurement was also performed with FSP920 spectrofluorimeter (*Edinburgh Instrument*) for all the cases. Steady-state anisotropy (r) was defined by

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad \text{(Eq. S1)}$$

Where, I_{VV} and I_{VH} are the intensities obtained with the excitation polarizer orientated vertically and the emission polarizer oriented vertically and horizontally, respectively. The *G* factor is defined as,

$$G = \frac{I_{HV}}{I_{HH}} \quad \text{(Eq. S2)}$$

The "*I*" terms refer to the parameters similar to those mentioned above for the horizontal position of the excitation polarizer and vertical and horizontal position of the emission polarizer, respectively. Time-resolved intensity decays of the enzyme at different state were measured using a Life Spec II spectrofluorimeter (*Edinburgh Instrument*). The sample was excited by Pico-quant 290 nm laser source, and the decay was measured in a time scale of 0.0488 ns/channel. The decay curves were analyzed by FAST software using the discrete exponential method Edinburgh Instrument along with the fluorescence instrument. The generated curves for intensity decay were fitted in the functions

$$I(t) = \sum_i \alpha_i \exp(\frac{-t}{\tau_i}) \quad \text{(Eq. S3)}$$

where α_i is the initial intensity of the decay component *i*, having a lifetime τ_i. The mean lifetime (τ_m) of trypsin in different experimental conditions was also calculated from following equation,[4]

$$\tau_m = \frac{\sum_i \alpha_i \tau_i}{\sum_i \alpha_i} \quad \text{(Eq. S4)}$$

Fourier transform infrared spectra. Trypsin molecules in native and in adsorbed state on CuS nanoparticles were lyophilized for FT-IR analysis. All the FT-IR spectra of lyophilized samples were measured with 1 mg samples in 200 mg of KBr as a pellet on the range of 2500−500 cm^{-1}. After background correction, Fourier self-deconvolution (FSD) was applied to the unsmoothed spectra for band narrowing (full width half-maxima, fwhm = 16 cm^{-1} and enhancement factor, $k = 2.0$).[5] Gaussian curve-fitting was then performed using GRAMS/AI software (*Thermo Fisher Scientific, Massachusetts*) on the Fourier self-deconvoluted amide I band region. The amide I region (1600−1700 cm^{-1}) of FT-IR spectrum has been widely used to quantify the individual elements of secondary structure of a protein in liquid as well as lyophilized samples.[6] The secondary structural content was calculated from the areas of the individual assigned bands and their fraction of the total area in the amide I region. In each case a linear baseline was fitted in addition to the Gaussian bands.

***Circular Dichroism*.** The secondary conformational characteristics of trypsin in native solution and after interaction with CuS nanoparticles were determined by far-UV CD spectra in a J-810 (*JASCO, Japan*) spectropolarimeter. An increased concentration from 0.5-5.0 mg mL^{-1} of CuS nanoparticles was incubated with fix amount of trypsin solution (0.1 mg mL^{-1}) to study the available nanoparticle surface area dependent secondary structural changes of trypsin. Spectra were recorded in a 0.1 cm path length quartz cuvette for acquisition in the far-UV region of 190−240 nm. For all the samples, data was collected by averaging three scans with the scan rate of 50 nm per min at 20 °C. The ellipticity was measured in millidegrees. The resolution and the time constant were 0.2 nm and 0.125 s, respectively. Blank spectra of the solution and of the particle suspension without enzyme were obtained at identical conditions for background correction. Circular dichroism spectra obtained from these experiments were further analyzed in web based DichroWeb server,[7] using CONTIN analysis program, which is based on nonlinear regression fitting algorithms without constraints. An average molar mass of 115 Da per amino acid residue was used for calculating the molar ellipticity, [θ].

Analysis of enzymatic activity of native and immobilized trypsin

The enzymatic activity assay of free and immobilized trypsin (at different surface density and temperature) was analyzed by using BAEE (*N*-α-benzoyl-L-arginin-ethyl ester) as substrate[8] in 50mM Tris-HCl buffer (pH 8.0). Typically, the enzymatic activity (U) represents the conversion of 1 μmoL of the substrate per minute, and the specific activity is defined as the enzymatic activity per mg of the enzyme (U mg^{-1}). However, here we present the relative specific activity (%) of immobilized enzyme compared to the same amount of the native enzyme in solution, for better understanding of the loss in enzyme activity upon adsorption. Same amount of native and adsorbed enzyme was incubated with desired concentration of substrate solution (BAEE) for a defined time duration and the concentration of the hydrolyzed product was measured. The relative retention of specific activity (%) by the immobilized enzyme molecules was calculated by taking the ratio of the amount of hydrolyzed product generated by the immobilized enzyme to the product generated by same amount of native enzyme. For the activity tests, 1 mM of BAEE solution was incubated with a fix amount of free trypsin and trypsin adsorbed nanoparticles, in a 1 mL vial under continuous stirring. The hydrolysis of BAEE by trypsin was determined spectrophotometrically at 253 nm by monitoring the increase of the reaction product (the esterolysis product is much more absorptive than BAEE at 253 nm) at each 5 min interval until saturation reaches. In case of immobilized trypsin, the enzyme loaded nanoparticles were separated from the solution in every 5 min by centrifugation at 15000 rpm. Thereafter, the supernatant was analyzed for

the concentration of the hydrolyzed product. The enzymatic activity of free and immobilized trypsin was also studied in a temperature range of 10-50^{o} C, using the temperature controlled system as mentioned before. Further, the enzyme kinetic study was performed by adding 0.2−1.5 mM BAEE to same amount of free and immobilized trypsin molecules. Michaelis-Menten kinetic model was fitted to the experimental data to get the enzyme kinetic parameters of native and immobilized trypsin.

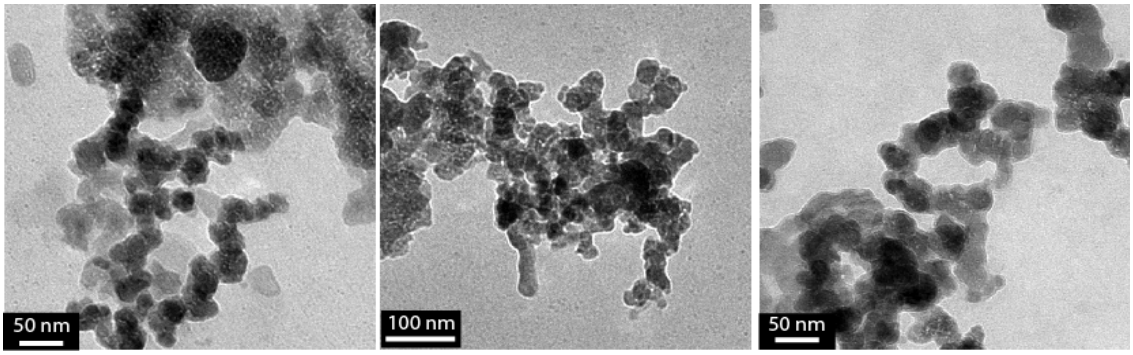

Fig. S1. Additional TEM images of CuS nanoparticles (20-50 nm).

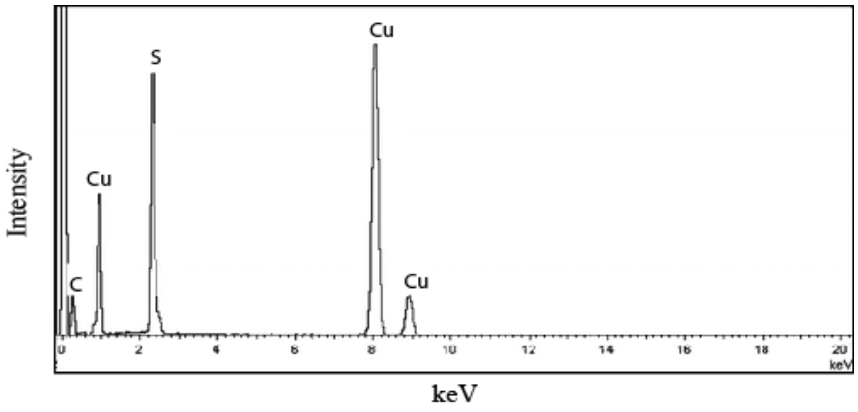

Fig. S2. Energy-dispersive X-ray (EDX) spectrum of synthesized CuS nanoparticles, showing the presence of Cu and S.

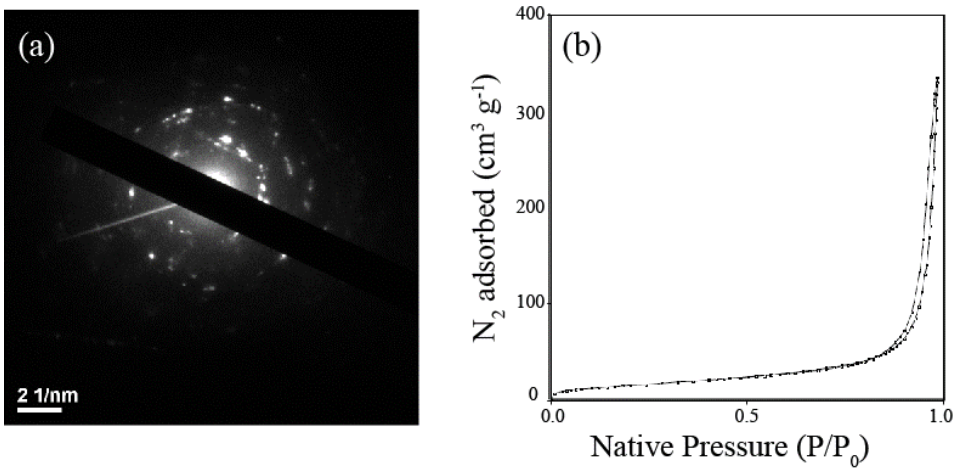

Fig. S3. (a) Selective area electron diffraction (SAED) of CuS nanoparticles; and (b) N_2 adsorption/desorption isotherms of CuS nanoparticles measured from BET analysis.

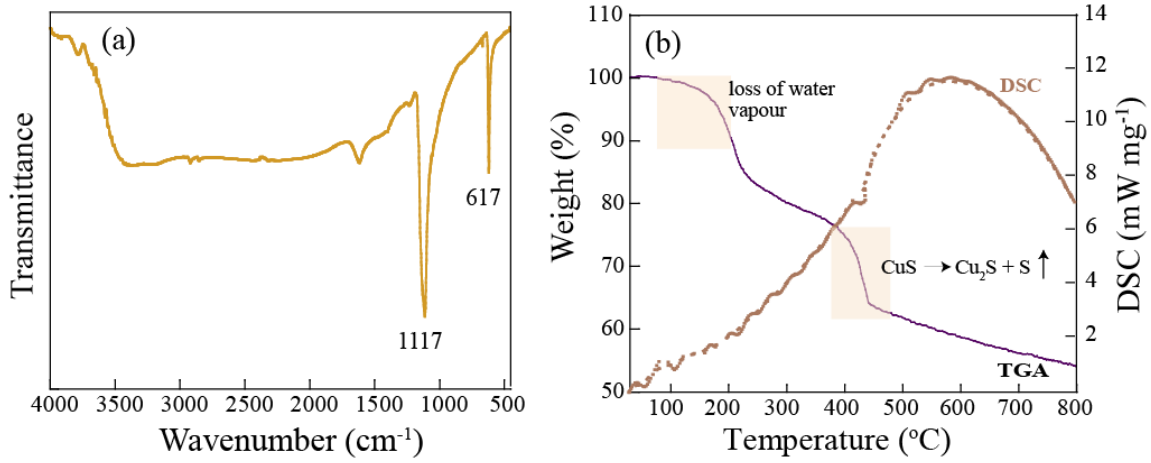

Fig. S4. (a) FT-IR spectra of CuS nanoparticle is taken in the range of 4000-450 cm^{-1}, with a scan number of 20 (scan resolution 4cm^{-1}); and (b) Thermogravimetric (TGA-DSC) analysis of CuS nanoparticles.

First order and second order kinetic model analysis on experimental kinetic data:

The linearized form of pseudo first order kinetic model (Eq. S1) and second order kinetic model (Eq. S2) are described as:

$$\log\left(q_e - q_t\right) = logq_e - \frac{k_1}{2.303}t \qquad \text{(Eq. S1)}$$

$$t/q_t = \frac{1}{k_2.q_e^2} + \frac{t}{q_e} \qquad \text{(Eq. S2)}$$

where q_t and q_e are adsorption capacity at time t and at steady state, respectively, and k_1 and k_2 are first order and second order kinetic rate constants, respectively. The linearized curve fit for both the kinetic model for trypsin adsorption is plotted in Fig. S5 and the kinetic parameter extracted from this fitting is given in table S1 below.

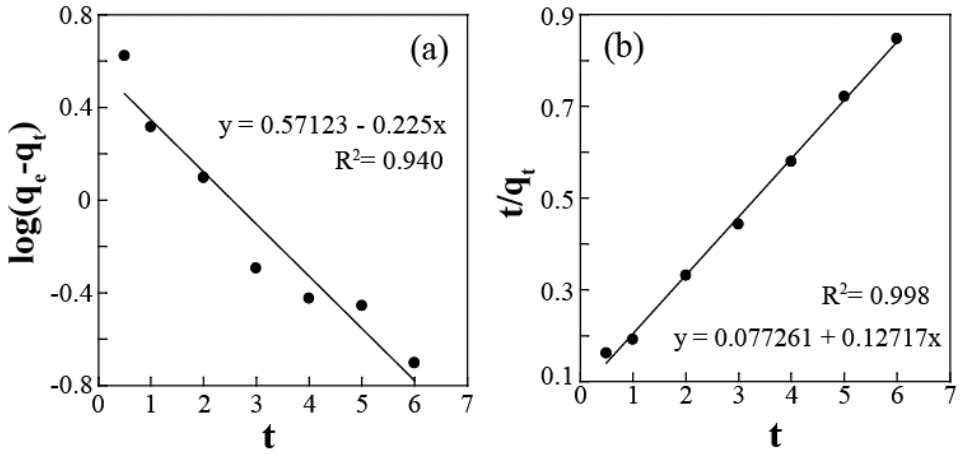

Fig. S5. Adsorption kinetics of trypsin on CuS nanoparticles analyzed by (a) first order and (b) second order kinetic model.

Table S1. Kinetic parameters of trypsin adsorption on CuS nanoparticles

	Experimental q_e *(mg m^{-2})*	**First order kinetics**			**Second order kinetics**		
		Calculated q_e *(mg m^{-2})*	k_1 *(L h^{-1})*	R^2	*Calculated* q_e *(mg m^{-2})*	k_2 *(m^2 mg^{-1} h^{-1})*	R^2

Trypsin	7.2	4.62	0.593	0.940	7.6	0.233	0.998

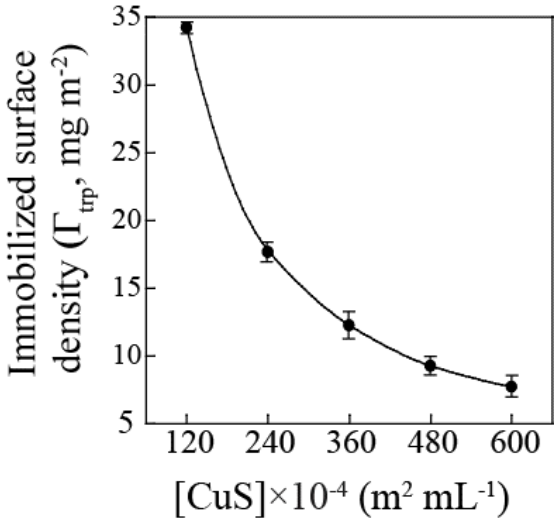

Fig. S6. Adsorption capacity of trypsin on CuS nanoparticles as a function of available nanoparticle surface area. Increasing amount of nanoparticle surface area for binding were made available (120×10^{-4} to 600×10^{-4} m^2 mL^{-1}), by adding 0.2 – 1.0 mg mL^{-1} of CuS nanoparticles to 0.1 mg mL^{-1} of trypsin solution.

Table S2. Time-resolved fluorescence (λ_{max} = 345 nm) spectroscopic parameters of trypsin interaction with CuS at increasing available nanoparticle surface area.

[CuS] (μg mL^{-1})	CuS (m^2 mL^{-1})	τ_1 (ns)	τ_2 (ns)	α_1	α_2	τ_m (ns)	χ^2
0 (native)	0	0.721	5.66	0.019	0.015	1.96	1.000
1	0.6×10^{-4}	0.610	3.48	0.022	0.015	1.77	1.000
3	1.8×10^{-4}	0.080	3.02	0.118	0.014	0.39	1.000
5	3.0×10^{-4}	0.028	2.93	0.375	0.011	0.11	1.000

Table S3. Secondary structural analysis of trypsin by circular dichroism after interaction with CuS nanoparticles at different concentration. Secondary structural content were determined by using Dichroweb software by curve fitting method.

CuS addition ($\mu g\ ml^{-1}$)	*CuS addition* ($m^2\ mL^{-1}$)	*α-helix(%)*	*β-sheet (%)*	*β-turn (%)*	*Random coil (%)*
0.0	0	7.2	41.8	12.2	39.6
0.5	0.3×10^{-4}	7.1	41.0	12.5	39.6
2.0	1.2×10^{-4}	7.0	40.8	12.4	39.8
3.0	1.8×10^{-4}	6.8	41.0	12.3	39.2
5.0	3.0×10^{-4}	6.7	40.8	12.1	39.2

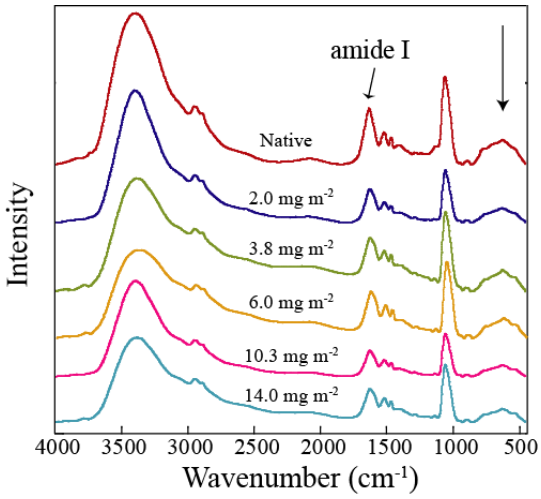

Fig. S7. FT-IR spectra of free trypsin and after interaction with CuS nanoparticles in 4000-450 cm^{-1} of range (Arrow indicates the Amide I region which has been further used for Gaussian distribution analysis). Top graph represents the FT-IR spectra of native trypsin, and the followings are the FT-IR spectra of immobilized trypsin at different surface coverage (mg m^{-2}).

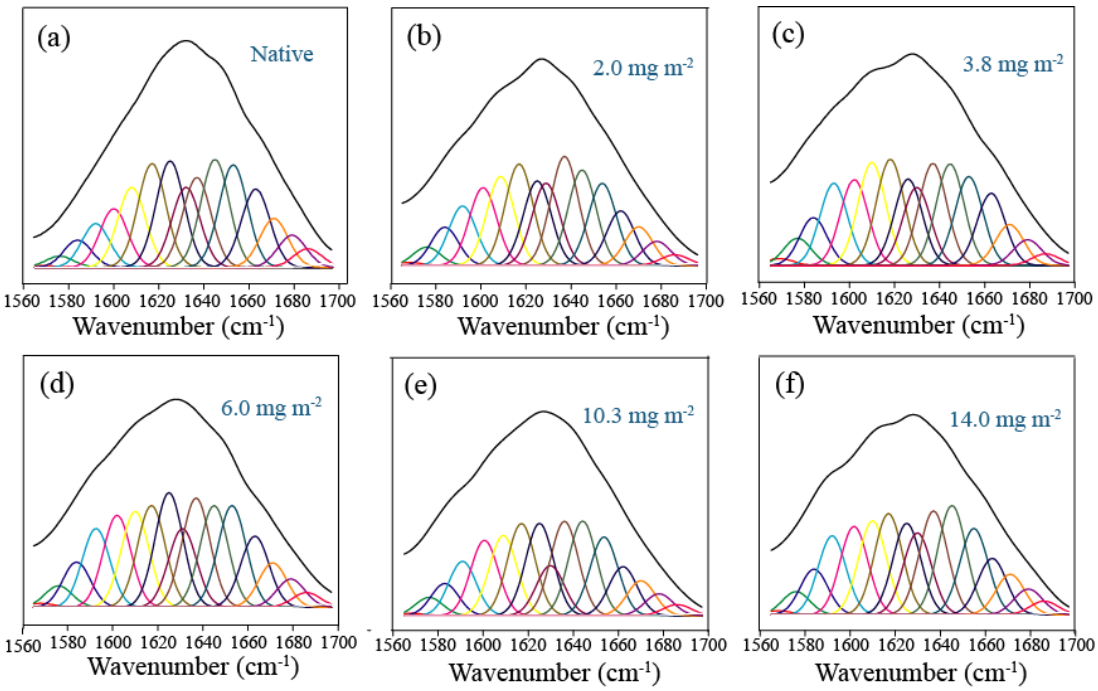

Fig. S8. Gaussian distribution analysis for secondary structural content of trypsin in (a) native state; and adsorbed on surface CuS nanoparticles at surface coverage of (b) ~2.0 mg m^{-2}, (c) ~3.8 mg m^{-2}, (d) ~6.0 mg m^{-2}, (e) ~10.3 mg m^{-2}, and (f) ~14.0 mg m^{-2} (for analysis of adsorbed trypsin, the FT-IR spectra of bare CuS was subtracted from the conjugate).

Table S4. Enzymatic activity values of immobilized trypsin on CuS nanoparticles at different surface density.

Surface density of trypsin (mg m^{-2})	Retention of Enzymatic activity (%)
1.9	98.3
3.5	92.1
6.0	89.2
7.4	86.7
10.3	81.3
14.0	77.5

Temperature controlled binding, conformation and activity of immobilized trypsin

Adsorption capacity and activity at different temperature. Interaction of trypsin with CuS nanoparticles was also studied in a temperature range of 10 ^{o}C to 40 ^{o}C by analyzing the binding capacity, thermodynamics as well as the structure-functional features. The surface density of immobilized trypsin scales down from around 30 mg m^{-2} at 10 ^{o}C to 14 mg m^{-2} at 40 ^{o}C (Fig. S9a). The decrease in surface coverage at higher temperature indicates the adsorption process is exothermic in nature; however the reason behind lower adsorption capacity at higher temperature is not well understood. However, the adsorption capacity of trypsin at room temperature (25 ^{o}C) from these set of experiments, should not be directly compared with the previous adsorption capacity data which was also performed at room temperature (Fig. 5a in main manuscript and Fig. S6), as different concentration of enzyme and nanoparticle was used for each set of experiments. A negative standard Gibb's free energy (DG^{o}) values for trypsin adsorption was obtained in the whole temperature range (-17 to -12 KJ mol^{-1}), which indicates the thermodynamic favourability of this adsorption process (Table S5). Moreover, the standard average enthalpy change (ΔH^{o}) and entropy change (ΔS^{o}) was calculated from the Van't Hoff equation. A linearized plot between $\ln(K_c)$ and 1/T was used to calculate the values of ΔH^{o} and ΔS^{o} (Fig. S9b). The average change in entropy (ΔS^{o}) for trypsin adsorption process was found to be -137.1 J K^{-1} mol^{-1}. The negative value of ΔH^{o} (-56 KJ mol^{-1}) also supports the exothermic nature of the adsorption process.

Further, the enzymatic activity of the same set of trypsin immobilized CuS nanoparticles, scales up with the temperature from 10 ^{o}C to 40 ^{o}C (Fig. S9a). In this case, the activity of immobilized trypsin was compared to the native enzyme activity which was also measured under same temperature. At 10 ^{o}C, the immobilized trypsin retains only ~40% of its native activity, which increases up to ~90% at high temperature of 40 ^{o}C; noticeably, native trypsin molecule is also reported to have its optimum activity around similar temperature (~37^{o} C).[3] Moreover, as the trypsin adsorption capacity was also varied during this temperature range (from 30 mg m^{-2} at 10 ^{o}C to 14 mg m^{-2} to 40 ^{o}C), therefore we interpret both the surface density of immobilized enzyme and the temperature might have a combine influence on the activity of immobilized trypsin in the temperature range studied here.

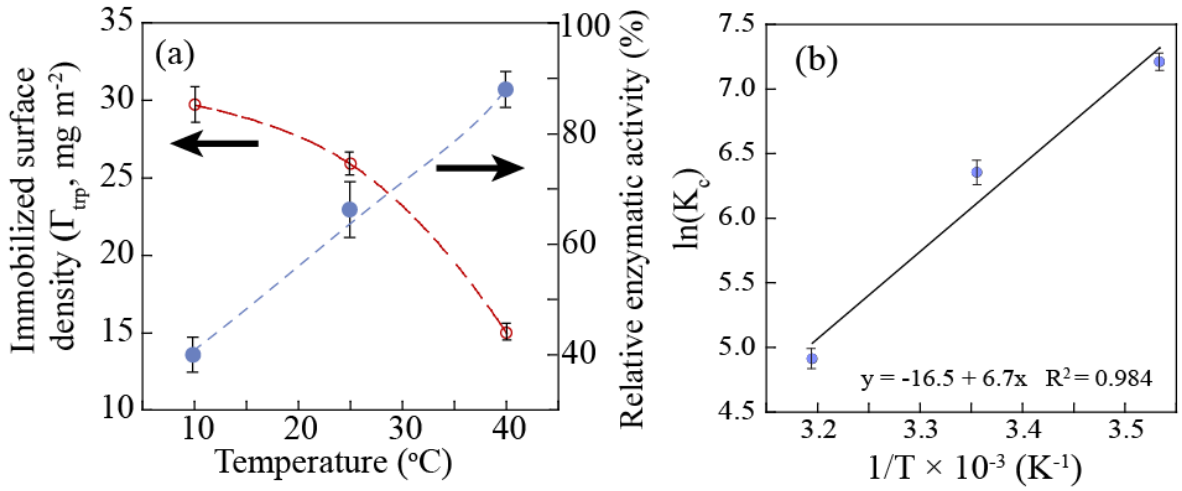

Fig. S9. Influence of temperature on trypsin interaction with CuS nanoparticles: (a) trypsin adsorption capacity (red) and enzymatic activity (blue) at different temperature. (b) Van't Hoff plot of trypsin molecules adsorbed on CuS nanoparticles. The Van't Hoff equation is expressed by: $ln(K_c) = -\frac{\Delta H}{R}\cdot\frac{1}{T} + \frac{\Delta S}{R}$. Where, K_c is the equilibrium constant, R is the universal gas constant, ΔH and ΔS are the average change in enthalpy and entropy respectively. The solid line represents the fit of the equation. From the slope and y-axis intercept of this plot, the average enthalpy change (ΔH^o) and entropy change (ΔS^o) was calculated and given in Table S5.

Conformation of immobilized trypsin at different temperature. Tertiary conformation of trypsin interaction with CuS as a function of temperature, was studied in the range of 25 °C to 50 °C using fluorescence spectroscopy. Concentration of trypsin and CuS was chosen to obtain a low surface density of immobilized trypsin (around 2.0 mg m^{-2}), which was found to have high retention of native enzyme conformation at room temperature from previous studies. The normalized fluorescence emission spectra of native and immobilized trypsin under a continuous increase of temperature are shown in Fig. S10.

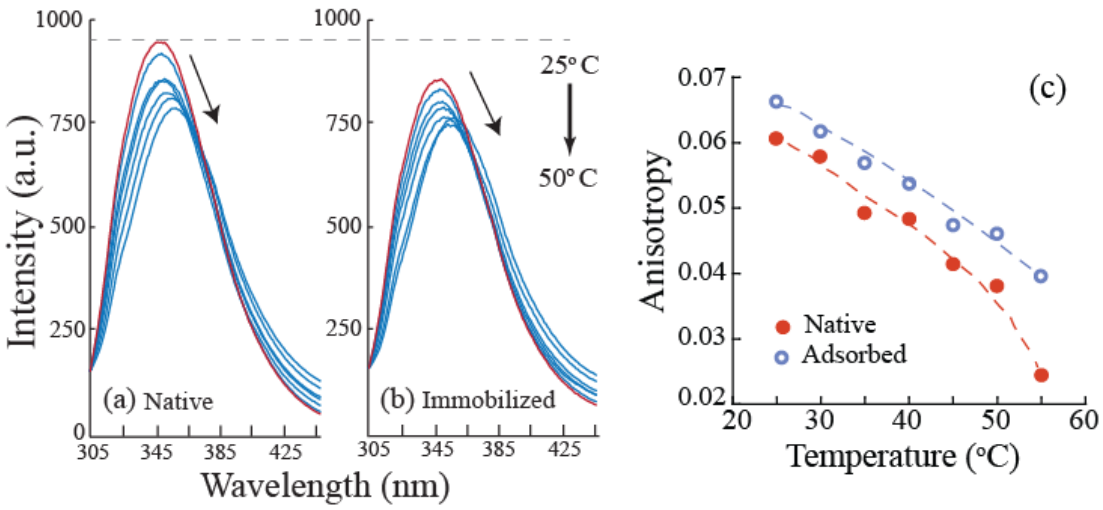

Fig. S10. Normalized steady state fluorescence emission spectra of (a) native (in solution) and (b) immobilized trypsin on CuS nanoparticles as a function of temperature (from 25 °C to 50 °C). The fluorescence spectrum in red colour represents the initial fluorescence emission of native and adsorbed trypsin at 25 °C. The dashed line represents the maximum emission intensity (λ_{max} = 345 nm) of native trypsin, for the sake of comparison with the emission spectra of immobilized trypsin at 25 °C. (c) Influence of temperature on trypsin conformation, as analyzed by steady state fluorescence anisotropy values in native and adsorbed state at different temperature. Dashed line are guide to the eye.

The initial λ_{max} (at 345 nm) value of immobilized trypsin at 25 °C was slightly lower than that of the native enzyme, indicating a partial loss in the tertiary structure of immobilized trypsin at room temperature, which is in agreement with the previous observations. In both the cases, upon increase in temperature, the emission intensity was quenched in a similar pattern with a small red shift. This specifies an increased alteration/unfolding in the tertiary structure of both native and immobilized trypsin molecules at higher temperature. The anisotropy of native and immobilized trypsin were also simultaneously measured in the temperature range of 25 °C to 50 °C. Unlike the previous cases (referring Fig. 2b and 5b in main manuscript), here the anisotropy values scale down with the increase in temperature for both native and immobilized trypsin (Fig. S10c). The decrease in anisotropy values can be attributed to the increased unfolding of protein tertiary structure at higher temperature.[9] Furthermore, the anisotropy values for immobilized trypsin were higher compared to the native enzyme throughout this temperature range, which is mainly due to the adsorption induced partial restriction of enzyme tertiary conformation.

Table S5. Thermodynamic parameters of trypsin adsorption on CuS nanoparticles.

Temp (^oC)	*ΔG (KJ mol^{-1})*	*ΔS (J K^{-1} mol^{-1})*	*ΔH (KJ mol^{-1})*
10	-16.9		
25	-15.7	-137.1	-56
40	-12.7		

References (from supporting information)

[1] S. P. Brunauer, P. H. Emmet and E. Teller, Adsorption of gases in multimolecular layers, J. Am. Chem. Soc., 1938, 60, 309-319.

[2] C. H. Yu, A. Al-Saadi, S. J. Shih, L. Qiu, K. Y. Tam and S. C. Tsang, Immobilization of BSA on Silica-Coated Magnetic Iron Oxide Nanoparticle, J. Phys. Chem. C, 2009, 113, 537-543.

[3] T. Sipos and J. R. Merkel, Effect of calcium ions on the activity, heat stability, and structure of trypsin, Biochemistry, 1970, 9, 2766–2775.

[4] R. Swaminathan, G. Krishnamoorthy and N. Periasamy, Similarity of fluorescence lifetime distributions for single tryptophan proteins in the random coil state, *Biophys. J.,* 1994, *67*, 2013-2023.

[5] K. Fu, K. Griebenow, L. Hsieh, A. M. Klibnov and R. Langer, FTIR characterization of the secondary structure of proteins encapsulated within PLGA microspheres, *J. Control. Release*, 1999, *58*, 357-366.

[6] K. Griebenow and A. M. Klibanov, Lyophilization-induced reversible changes in the secondary structure of proteins, *Proc. Natl. Acad. Sci. U.S.A.,* 1995, *92*, 10969– 10976.

[7] L. Whitmore and B. A. Wallace, DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data, *Nucleic Acids Res*., 2004, *32*, W668-673.

[8] G. W. Schwert and Y. A. Takenaka, Spectrophotometric determination of trypsin and chymotrypsin, *Biochim. Biophys. Acta,* 1995, *16*, 570-575.

[9] M. R. Eftink, The use of fluorescence methods to monitor unfolding transitions in proteins, *Biophys. J*., 1994, *66*, 482-501.