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

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Abstract

The biological activity of immobilized enzyme is central to the performance of different nanoparticle mediated enzymatic assays, where enzymatic conversion can be used for label-free analyte detection. In this article we have addressed two significant aspect of enzyme-nanoparticle interaction. First, we have developed copper sulfide (CuS) nanoparticles, with an average diameter of 25 nm, as a potential enzyme-interface, using trypsin protease as a model enzyme. CuS nanoparticles showed high trypsin immobilization capacity up to 14.0 mg m^{-2} with significant retention of native enzymatic activity (75%-98%) in room temperature, even beyond calculated tightly packed monolayer coverage (which is around 4.1 mg m^{-2}). Second, we report a quantitative correlation between the structure-functional relationship and the density of immobilized trypsin on nanoparticle surface. The in-situ conformation of immobilized trypsin could be analyzed well by fluorescence, circular dichroism and FT-IR spectroscopic measurements, due to the small size of the nanoparticles. Trypsin molecules appear to retain their close-native tertiary and secondary structural features (with a small loss of 1-2% of helical content) in the entire surface density range (2.0 – 14.0 mg m^{-2}) on CuS nanoparticles. However, interestingly, at low surface coverage (2.0 mg m^{-2}), immobilized trypsin retains almost 98% of its native enzymatic activity, leading to a highly functional bio-nanocomposite. While at higher surface coverage, the enzyme activity decreases to 77%, indicating the influence of steric crowding. Further, the high functionality of immobilized trypsin at low surface density on CuS nanoparticle was also supported by extracting the kinetic parameters of enzymatic activity.

Introduction

Recent development in bio-nanotechnology have a large socioeconomic impact in bio-medical industrial sectors, as the use of nanomaterials is constantly increasing in the industrial activities such as in biosensing, diagnostics, biomedicine and therapeutics.[1,2] The interaction of protein/enzyme molecules with nanomaterials is the core of such applications; where the surface activity of immobilized proteins/enzymes is crucial for the assembly of interfacial protein constructs.[3,4] However, not much is investigated on how different factors collectively influence the activity of immobilized enzyme, especially on nanoparticle surface. Upon interaction with nanoparticles, enzyme molecules may alter its conformation, expose new epitopes on the protein surface, or even get perturbed from its normal function.[5] In general, different parameters can influence the conformation and the surface activity of immobilized enzymes likely, immobilization techniques and physico-chemical parameters, surface charge of nanoparticle and the enzyme molecules, surface packing density of immobilized enzymes, roughness of the nanoparticles etc. Surface density of immobilized enzyme molecules can be a significant parameter in terms of achieving desired enzyme activity on nanoparticle surface, as molecular crowding on surface can directly influence enzyme conformation and subsequently its activity. Although there are reports on different immobilization techniques to achieve high enzyme activity,[3] the in-depth correlation between the important parameters likely the activity, conformation and the density of the enzyme molecules, is still not well established especially on nanoparticle surface. It will be of great scientific interest to know this correlation of enzyme density, structure and function at nano-interface under relevant physico-chemical conditions, for real-time applications such as bio-sensing or proteomics.

The choice of nanomaterials as enzyme carrier is another important aspect for immobilized enzyme activity. Surface area, roughness and chemical groups of nanomaterials play an important role in interaction with enzyme molecules. In this regard, we have chosen the sulfur based nanomaterial, copper sulfide (CuS), for interaction of enzyme molecules. Copper sulfides exist as a range of stable and metastable phases ranging between copper 'poor' CuS (covellite) to copper 'rich' Cu_2S (chalcocite) at

room temperature, common Cu_xS phases are $Cu_{1.75}S$(anilite), $Cu_{1.8}S$(digenite) and $Cu_{1.94}S$ (djurleite).[6,7] Although copper sulfides has been studied widely in semiconductors, solar cell materials, and as catalyst,[8-11] it has not been studied extensively as bio-interface. Presence of sulfur on the surface can make it a suitable tool to interact with biomolecules, which can bind effectively with the thiol based amino acid side groups (like cysteine) of a protein. Some recent studies have also indicated the possibility of using CuS as a biomolecular support, facilitating different biological applications such as cancer cell cytotoxicity,[12] tissue imaging[13] etc. Moreover, the catalytic property of CuS can be utilized itself as a label for detection, which will be interesting for developing 'label-free' biosensing assays. In a recent study, Zhu *et al*. have demonstrated a sensitive biosensing of prostate specific antigen (up to 0.1 pg mL^{-1}), using target specific antibody coated CuS nanoparticles as a label.[14] These results reinforce the scientific interest to investigate in-depth interaction features of protein molecules with CuS nanoparticles, from which we can extrapolate suitable parameters for developing functional bio-nanocomposites with desired activity for above mentioned biotechnological applications. Herein, we have used the trypsin protease as a model protein for the interaction with CuS nanoparticles. Trypsin is a medium-sized globular protein which itself has a significant biotechnological applications such as in proteomics, tissue culture, food processing and nanomedicine.[15-17] Trypsin cleaves peptide bonds at the carboxylic groups of arginine, lysine, and ornithine, and works optimally at pH 7.5-8.5.[18]

In this article we have addressed two important aspect of protein-nanoparticle interaction. First, we have studied how the surface density, conformation and activity of immobilized enzyme molecules interrelates quantitatively on a nanoparticle surface; and second, we have demonstrated the potential application of CuS nanoparticle as an enzyme carrier, with high retention of enzymatic activity facilitated by simple physisorption. Physorption of enzymes can discard the requirement of any additional chemical modification/labeling of the protein molecules for immobilization, which facilitates a much simpler strategy for protein immobilization. Trypsin molecules were physisorbed on CuS nanoparticles at different density and different physico-chemical parameters (*viz.* available surface area and temperature), and subsequently subjected to conformational and activity analysis. Due to smaller

hydrodynamic diameter of CuS nanoparticles, it does not significantly scatter or absorb light, therefore we could directly analyze the in-situ conformation and activity of immobilized trypsin using different spectroscopic techniques. Further we have also extracted the kinetic parameters to support the high functionality of immobilized trypsin in situ, by using the standard Michaelis-Menten kinetic model.

Experimental

Materials. $Cu(CH_3COO)_2$, pyridine-*2*,*3*-dicarboxylic acid (PDA), Tris and all the other components used for buffer preparation, were purchased in analytical grade from *Merck India Ltd*.

Enzyme. Lyophilized trypsin from porcine pancreas (1:250, crystal structure is illustrated in Scheme 1a, 1b) and its substrate N_α-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE) were purchased from *Sigma Chemicals*, USA. Prior to each experiment, fresh aqueous enzyme solutions of different concentrations were prepared in 10 mM Tris-HCl buffer (pH 8.0) containing 10 mM of $CaCl_2$. The use of $CaCl_2$ helps to prevent the autolysis of the enzyme as well as maintain its high activity. All the solvents used in this study were of spectroscopy grade and the distilled water and deionized water (Milli-Q system at 18.2 MΩ, Millipore, USA) was used in all experiments.

Synthesis and characterization of CuS nanoparticles. CuS nanoparticles were prepared via passing H_2S through a copper-acid complex solution. Briefly, aqueous solution of $Cu(CH_3COO)_2$ and pyridine-*2*,*3*-dicarboxylic acid (PDA) was mixed in a molar ratio of 1:2 to form a Cu-PDA complex, which readily precipitates out from the solution. This Cu-PDA complex was used as precursor template to synthesize ultrafine CuS nanoparticles. The precipitated complex was further washed and re-dissolved into basic aqueous medium by slow and continuous addition of piperidine. H_2S gas was then passed through this Cu-PDA complex solution in a controlled manner under vigorous stirring for 30 min, and stirred additionally for 30 min. A colloidal greenish-black suspension was formed by this process, which was separated by high speed centrifuge at 15000 rpm for 15 min. Precipitate was further re-suspended with Milli-Q water and methanol to wash several times for removing the impurities. Finally, the washed

CuS nanoparticles were collected after drying under vacuum and used for further interaction studies with trypsin.

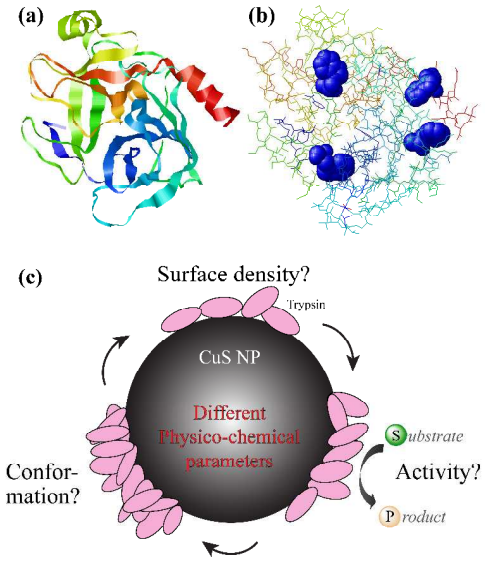
Synthesized nanoparticles were characterized in transmission electron micrograph (TEM), scanning electron microscope (SEM), FT-IR, powder XRD and thermogravimetric analysis (TGA). Surface area of the nanoparticles were measured by BET procedure by measuring the N_2 adsorption. The detailed characterization techniques are given in the supporting information.

Adsorption of trypsin. Batch adsorption experiments of trypsin on CuS nanoparticles were performed at pH 8.0 (with 10 mM Tris-HCl buffer), at a function of time, system temperature, and trypsin and CuS nanoparticle concentration. The amount of adsorbed enzyme was calculated by measuring the supernatant trypsin concentration using Bradford assay and measuring the absorbance at 280 nm. The detailed adsorption experiments of trypsin-CuS interaction and calculations are described in the supporting information, and summarized in Scheme 1c.

Conformational analysis of trypsin interaction. The tertiary conformation of native trypsin and immobilized at different physico-chemical parameters were measured using fluorescence emission spectroscopy. Trypsin-CuS interaction was also analyzed by measuring the fluorescence anisotropy and time-resolved fluorescence for understanding the adsorption induced molecular alteration of trypsin molecules. Further, we have analyzed the secondary conformation of immobilized trypsin as a function of available nanoparticle surface area and immobilized trypsin density, using circular dichroism and Fourier transformed infrared (FT-IR) spectroscopy. These experimental details and data analysis methodologies are described in detail in the supporting information.

Enzymatic activity of 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[19] in 50mM Tris-HCl buffer (pH 8.0). Here we present the relative specific activity (%) retained by immobilized enzyme compared to the same amount of the native enzyme in solution, for better understanding of the loss in enzyme activity upon adsorption. Further, the enzyme kinetic study was performed by adding different amount of BAEE (0.2−1.5 mM) and by analyzing using Michaelis-

Menten kinetic model. The detailed experimental procedure of enzyme activity kinetics is described in supporting information.



Scheme 1. Crystal structure of trypsin molecule: (a) distribution of α-helical and β-sheet elements as obtained from crystallographic data (http://www.rcsb.org). (b) Blue space-filled indicate the location of four tryptophan moieties in the three dimensional structure of trypsin molecule. (c) Brief schematic illustration of the experiments performed in this study: the trypsin molecules were immobilized on CuS nanoparticles at different physico-chemical conditions and the in-situ correlation of immobilized surface density, conformation and activity has been established.

Result and Discussion

Characterization of CuS nanoparticles. Transmission electron microscopic (TEM) images (Fig. 1a) and corresponding particle size distribution analysis (Fig. 1b) revealed the near-spherical shape of synthesized CuS nanoparticles with a size distribution in the range of 20−50 nm, which is fairly polydisperse. One control experiment has been also done by passing the H_2S gas to only $Cu(CH_3COO)_2$ solution, which resulted in bulk precipitation of CuS from solution. Due to the strong coordinating ability of carboxylate groups of PDA, it can bind strongly with metal center of Cu^{2+}.[20,21] Therefore, the bi-dentate ligand PDA acted as a stabilizing agent in preparation of CuS nanoparticle. The elemental

analysis of CuS nanoparticles with energy dispersive X-ray (EDX) spectra reveals the presence of only Cu and S in the CuS nanoparticle samples (Supporting Information, Fig. S2).

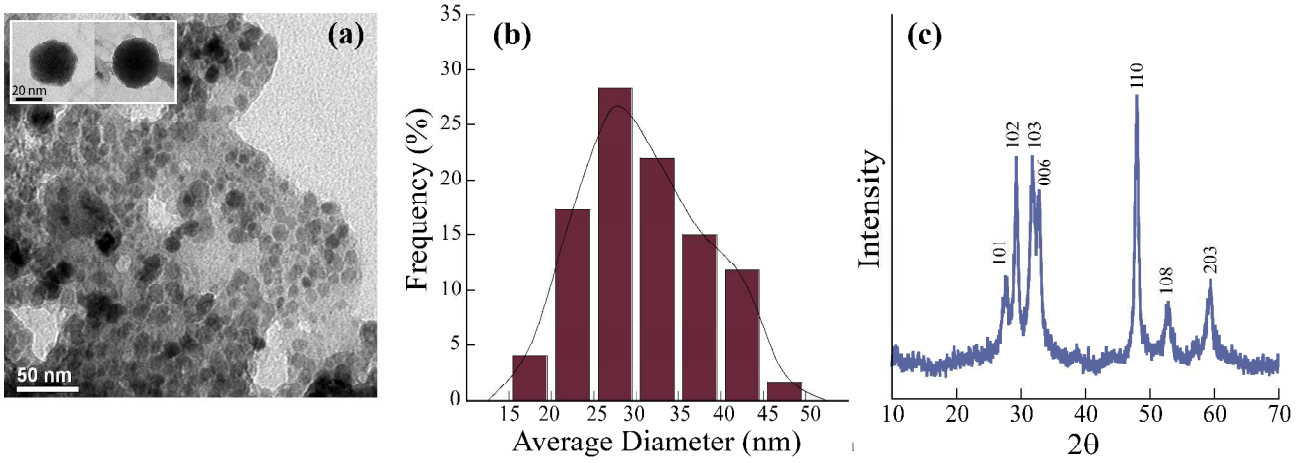


Fig. 1. Characterization of CuS nanoparticles: (a) TEM images (inset: individual nanoparticles) (b) size distribution analysis and (c) powder XRD (PXRD) spectra of synthesized CuS nanoparticles.

All peaks of the powder X-ray diffraction (PXRD) data shows the formation of crystalline nanoparticle (Fig. 1c), and the peaks are matching well with the covellite phase of CuS (JCPDS file no. 00-006-0464).[22] The crystalline nature of the CuS nanoparticles was also confirmed from the SAED analysis of the TEM images (Supporting information, Fig. S3a). The average crystallite size of CuS nanoparticles was calculated ~25 nm, using the Debye−Scherrer equation from the fwhm of the strongest peak, is consistent with the TEM and size distribution analysis. The N_2 adsorption isotherm of the BET analysis represents the type II isotherm according to Brunauer's classification (Supporting Information, Fig. S3b). From analysis, we found that CuS nanoparticles have a high specific surface area of 60 $m^2 g^{-1}$ and a pore volume of 0.449 $cm^3 g^{-1}$. FT-IR spectra (Supporting Information, Fig. S4a) show the characteristic peaks of CuS. The weak metal-sulfur band of CuS nanoparticles could be observed at ~617 cm^{-1} region.[23]

Thermal decomposition of CuS nanoparticles (in N_2 atmosphere), in the range of 25-100 ^oC was also studied (Supporting Information, Fig. S4b). At temperature range 100-150 ^oC the observed weight loss is mainly due to the release of water vapour associated with the material. The CuS nanoparticles undergo a desulfurization above 400 °C to give the product Cu_2S. Therefore, a further weight loss was

observed in the temperature range of 300-450 ^{o}C, which is due to this decomposition of CuS to Cu_2S and release of sulfur that occurred up to 430 ^{o}C (Eq. 1).[24]

$$2CuS \rightarrow Cu_2S + S\uparrow \quad (1)$$

This decomposition pattern is in good agreement with the previous reports.[25] Moreover; the DSC pattern (Supporting Information, Fig. S4b) of CuS nanoparticles in this temperature range also reflects a change in phase from CuS to Cu_2S at this temperature range. The theoretical and measured (from TGA graph) weight loss of CuS nanoparticles based on Eq. 1, was found similar (16.8% and 16.6% respectively), which supports the chemical characteristics of formation of CuS nanoparticles.

Time dependent interaction of trypsin with CuS nanoparticles. The kinetics of trypsin interaction with CuS nanoparticles (at pH 8.0, with 0.5 mg mL^{-1} CuS and 1.0 mg mL^{-1} trypsin) was studied for a duration of 6 h, and the time dependent adsorption capacity of trypsin is shown in Fig. 2a. Initially the adsorption capacity of trypsin scales up fast, leading to a plateau beyond 2 h, and thereafter reached steady state around the adsorption capacity of 7.0 mg m^{-2} within 4-5 h. More than 80% of the final capacity of trypsin was adsorbed within first 1 h. On a 'soft' surface (according to HSAB principle[26]) like CuS, binding of proteins may principally govern by electrostatic interactions. Previous studies reported the surface potential analysis (ζ-potential) of different CuS materials.[27] Covellite phase CuS nanomaterials was found to have an isoelectric point (IEP) at around pH 3.0. On the other hand, the IEP of molecular trypsin is around pH 9.0. Therefore, at pH 8.0 the CuS surface becomes negatively charged and the trypsin molecule is partially protonated, which can lead to a strong electrostatic bonding and resulted in high adsorption capacity for trypsin. In addition with electrostatic interaction, physisorption of trypsin can also govern via formation of hydrogen bonds between the protein molecules and nanoparticle surface. Moreover, having the sulfur group on the CuS surface may also facilitate the enzyme immobilization via di-sulfide bonds using the side chains of thiol containing amino acid of trypsin molecule like cysteine.[28]

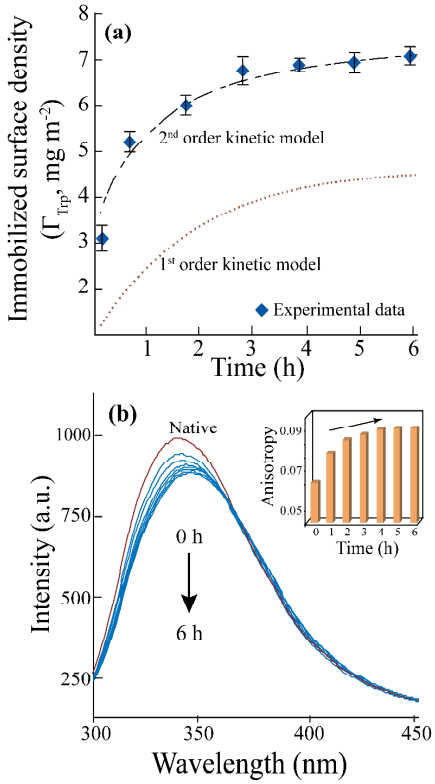


Fig. 2. Time dependent interaction of trypsin with CuS nanoparticles: (a) Immobilized trypsin density on CuS nanoparticles as a function of time. First order and second order kinetic equation model have been fitted (two different dotted lines) with the experimental data to extract the kinetic parameter of adsorption. (b) The steady state fluorescence emission spectrum in red represents the native trypsin's emission intensity, and the subsequent blue spectra represents the fluorescence emission intensity upon interaction with CuS nanoparticles as function of time; (inset) anisotropy values of trypsin interaction with CuS nanoparticles as a function of time.

First order and second order kinetic model was fitted with the experimental kinetics data of trypsin adsorption on CuS nanoparticles, and the kinetic parameters were calculated for both cases (Supporting Information, Fig. S5, Table S1). Second order kinetic model showed a higher correlation coefficient (R^2) of 0.998 than that of first order model ($R^2 = 0.940$), which can be attributed to the actual heterogeneous distribution of CuS surface. The experimental data were compared together with pseudo first and second

order models (Fig. 2a). The second order model matches well with the experimental data, while the first order model was far underestimated the actual amount of trypsin immobilized.

Further, the tertiary conformation of immobilized trypsin on CuS nanoparticles as a function of time was analyzed with steady state fluorescence spectroscopy. Trypsin molecules contain four tryptophan (Trp) moieties that can be used as intrinsic fluorophores (Scheme 1b). The λ_{max} of native trypsin emission spectrum in solution is characterized at 345 nm which indicates that specific tryptophan(s) of trypsin are partly exposed to the aqueous solvent (Fig. 2b). This observation is consistent with the reported native conformation of trypsin in solution.[29] Upon binding on CuS surface, the fluorescence intensity of trypsin quenched partially with time, with a small red shift (Fig. 2b).

Partial decrease in fluorescence intensity is possible when the tertiary conformation of adsorbed trypsin molecules alter in such a way that the Trp moieties occupy a positions in close proximity of other fluorescence-quenching amino acids. Among different amino acids, cysteine (Cys) is strong quenchers of tryptophan fluorescence and, therefore, in the native state tryptophan moieties neighboring to the Cys residues do not significantly contribute to the overall fluorescence emission.[30] In trypsin's three dimensional molecular structure, different Cys residues are in close proximity of the three Trp moieties (Trp 141, Trp 215 and Trp 237) of the enzyme. Only Trp 51 locates relatively far from the cysteine group, which therefore can contribute the majority of the fluorescence intensity.[30,31] However, the quenching of the fluorescence intensity slows down with time and saturates once steady state reaches (spectra from 4-6 h). This indicates upon initial binding on nanoparticle surface, trypsin molecules adopt partial loss in its tertiary conformation, and did not underwent total denaturation or drastic conformational change. Therefore, extensive molecular rearrangements of the trypsin molecule are not likely to be the major driving force for strong adsorption of trypsin on CuS surface. The displacement of trypsin molecule from CuS surface (after reaching steady state), has also been studied by re-dispersing the enzyme loaded nanoparticles into the enzyme-free buffer (10 mM Tris-HCl buffer) solution. A time dependent study up to 2 h showed no detectable trypsin molecules in the solution as obtained from the

Bradford assay and absorbance at 280 nm, which indicates a strong and stable interaction of trypsin molecules with CuS surface.

The fluorescence anisotropy of trypsin binding on CuS surface as a function of time has also been measured (Fig. 2b, inset). The anisotropy value of native trypsin in solution was found 0.064 which increases up to 0.085 upon binding on CuS surface. Increase in anisotropy values indicates the increased rigidity in the surrounding environment of the fluorophores upon binding on CuS surface. The increased anisotropy value can be attributed to the imposed motional restriction on the different fluorophores of trypsin, due to the adsorption. However, the anisotropy value saturates after adsorption capacity reaches steady state. This indicates no significant alteration in three dimensional structures further, which is a similar observation compared to previous steady state fluorescence analysis.

Binding and conformational analysis of trypsin at different available nanoparticle surface area. Binding of trypsin on CuS surface was studied at increasing available nanoparticle surface area, by adding different concentration of CuS nanoparticles to trypsin at room temperature. An increasing concentration of CuS nanoparticles (0.2 – 1.0 mg mL^{-1}) was added to a fixed amount of trypsin (0.5 mg mL^{-1}), to generate a range of available surface area from 120×10^{-4} to 600×10^{-4} m^2 mL^{-1}. The available surface area for trypsin binding was calculated approximately from the nanoparticle concentration (mg mL^{-1}) and its specific surface area (m^2 g^{-1}). The actual surface area can vary from this calculated values, due to the size distribution, and/or partial aggregation of nanoparticles locally. The immobilized surface density of trypsin (Γ_{trp}) scales down from 34.2 mg m^{-2} to 7.7 mg m^{-2} with the increase in available nanoparticle surface area up to 600×10^{-4} m^2 mL^{-1} (Supporting Information, Fig. S6).

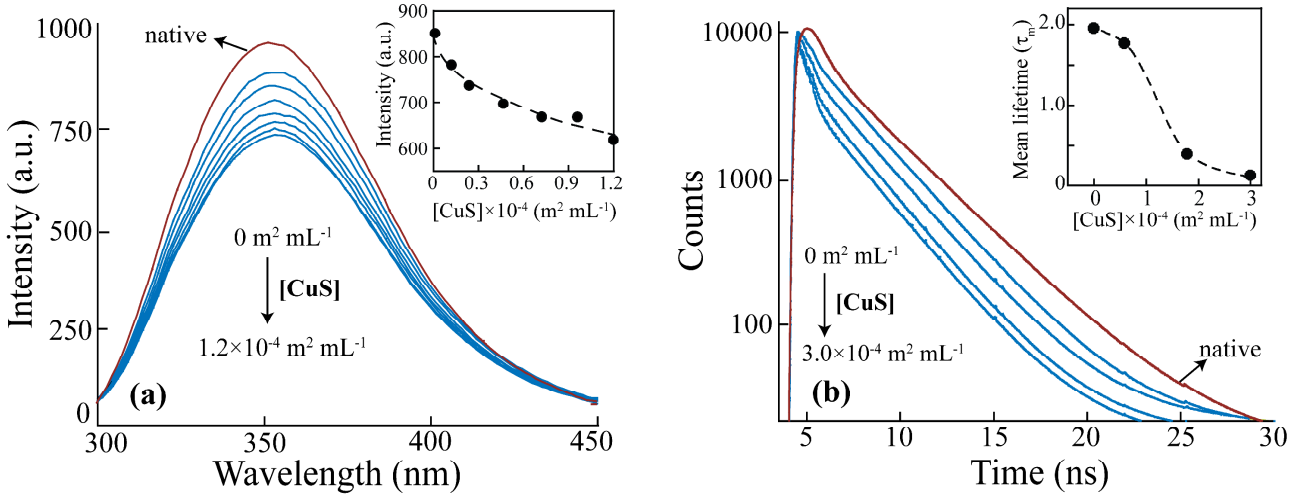


Fig. 3. Fluorescence analysis of trypsin-CuS binding as a function of total available nanoparticle surface area: (a) Steady state fluorescence spectroscopy showed quenching of emission intensity with the increase of CuS nanoparticle concentration. (inset) Maximum emission intensity (λ_{max}) at 345 nm as a function of available CuS nanoparticle surface area. (b) Time resolved fluorescence spectra of trypsin interaction with increasing CuS concentration. (inset) Calculated mean lifetime (τ_m) of trypsin molecules as a function of available CuS nanoparticle surface area.

Steady state and time resolved fluorescence spectra. Tertiary conformation of immobilized trypsin as a function of total available nanoparticle surface area was also studied using steady state and lifetime fluorescence spectroscopy. A lower CuS nanoparticle concentration range was used (0 – 5 μg mL^{-1}, corresponding up to 3×10^{-4} m^2 mL^{-1}) for the fluorescence analysis of trypsin-CuS binding, compared to the binding capacity studies (120×10^{-4} to 600×10^{-4} m^2 mL^{-1}, as described in the previous paragraph); which is mainly to minimize the optical scattering/absorbance by CuS nanoparticles at higher concentration. With the increase in available surface area (0 to 1.2×10^{-4} m^2 mL^{-1}), the steady state fluorescence intensity quenches partially (Fig. 3a), which indicates a partial unfolding of trypsin tertiary structure upon increasing concentration of CuS. A plot of λ_{max} (at 345 nm) of Trp emission spectra as a function of available CuS surface area shows an almost linear decrease in fluorescence emission

intensity of immobilized enzymes with the increase in available nanoparticle surface area (Fig. 3a, inset).

Time resolved fluorescence spectroscopy of trypsin molecules, upon interaction with CuS nanoparticles was analyzed by measuring the fluorescence lifetime of the Trp moieties as a function of total available nanoparticle surface area. The time-resolved decay of trypsin was taken at an emission wavelength of around 345 nm ($\Delta\lambda = \pm 5$ nm). The sums of two exponential decay model was fitted well with the fluorescence lifetime data, with a χ^2 value close to 1.00 (utilizing Eq. S3, Supporting information). The time resolved decay profiles of native trypsin was changed progressively upon interaction with the increase in available surface area for binding (Fig. 3b). The individual lifetime values (τ_1 and τ_2) and the amplitudes (α_1 and α_2) were calculated[32] from the bi-exponential fit (Supporting Information, Table S2), in addition with the mean lifetime values (τ_m) which was calculated using Eq. S4 (supporting information). The mean lifetime (τ_m) of native trypsin is close to 2 ns which scales down up to 0.11 ns upon interaction with the increased available nanoparticle surface area up to 3.0×10^{-4} m^2 mL^{-1} (Supporting Information, Table S2). At higher available surface area ($> 0.6\times10^{-4}$ m^2 mL^{-1}), the mean fluorescence lifetime decays rapidly (Fig. 3b, inset). The decrease in mean lifetime values (τ_m) of the fluorophore can be attributed to the alteration of polarity of the environment surrounding the fluorophore; which indicates partial unfolding of trypsin and exposure of fluorophore to the aqueous solvent upon binding on CuS nanoparticle surface.

Circular dichroism spectra. Secondary conformation of trypsin interaction as a function of available CuS nanoparticle surface area have been investigated by far circular dichroism (CD) spectroscopy. The CD spectra of native and immobilized trypsin exhibits a broad negative band having minimum molar ellipticity in the range of 200-208 nm with a shoulder at ~220 nm (Fig. 4). This is the characteristics of the enzyme like trypsin, which contains both α-helices and β-sheets.[33] Analysis of CD spectra reveals that native trypsin in solution contains ~7.2% α-helices, ~41.8% β-sheets and the rest ~51.8% of turn and other unordered elements. Although having lower signal to noise ratio, a similar shape of the CD

spectra of immobilized trypsin was observed compared to that of free enzyme. Analysis with Dichroweb software reveals a comparable secondary structural elements of the immobilized trypsin with its native form, after interaction with low concentration of CuS nanoparticles ($\leq 1.2\times10^{-4}$ m^2 mL^{-1}, Supporting Information, Table S3).

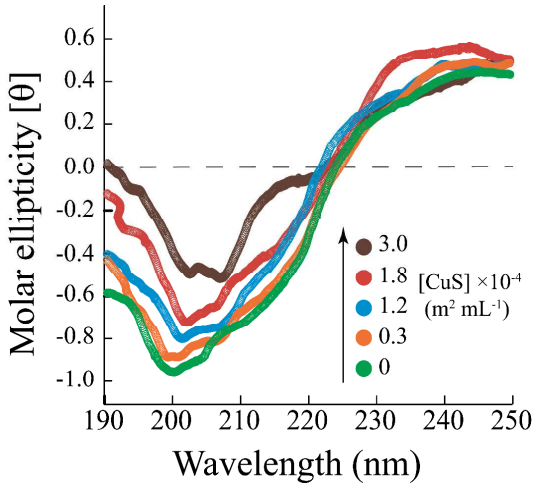


Fig. 4. Circular dichroism spectra of trypsin interaction with CuS nanoparticles as a function of increasing available nanoparticle surface area (by adding increased concentration of CuS nanoparticle).

At higher available nanoparticle surface area of CuS ($\geq 2.0\times10^{-4}$ m^2 mL^{-1}), the α-helical content of immobilized trypsin was decreased partially to ~6.7%. This set of data indicates that, upon interaction with CuS nanoparticles, although the tertiary conformation is partially rearranged, it marginally affects the secondary structural elements. Later in this article, we have also presented the secondary structural elements of immobilized trypsin as a function of its surface density on CuS nanoparticles.

Surface density of immobilized trypsin on CuS nanoparticles and structure-functional correlation

Surface density of immobilized trypsin: Isotherm studies at different surface density of immobilized trypsin on CuS surface was performed by adding a fix amount of CuS (1 mg mL^{-1}, corresponding to 60×10^{-4} m^2 mL^{-1}) to different concentration of trypsin (0.1 – 1.0 mg mL^{-1}) at pH 8.0 in room temperature. The steady state adsorbed amount of trypsin on CuS surface scales up with increasing initial trypsin concentration as shown in Fig. 5a. The initial high slope in the isotherm plot indicates

high affinity of trypsin for CuS surface, which approaches towards a plateau at higher trypsin concentration. Using the molecular dimensions of trypsin, the maximum theoretical surface concentration could be calculated. On the basis of crystallographic information (Protein Data Bank entry 2PTN),[29] the spatial conformation of the native trypsin molecule can be approximated as an ellipsoid with dimensions of 4.8 nm × 3.7 nm × 3.2 nm (Scheme 1a, 1b). Considering that the enzyme molecules are surrounded by a hydration layer of approximately 0.2 nm, and that they adsorb in a *side-on* and *end-on* configuration, a tightly packed monolayer would contain around 2.4 mg m^{-2} and 4.1 mg m^{-2} of unperturbed trypsin molecules respectively. However, the actual surface roughness of the CuS nanoparticles will lead to a different effective surface area per nanoparticle, which can shift these numbers to somewhat higher values. However, the isotherm data reveals that in the added concentration range of trypsin, the surface density of immobilized trypsin varies from a close-monolayer coverage (~2.0 mg m^{-2}) to a crowded tightly packed coverage (> 5 mg m^{-2}). With higher trypsin concentration, the adsorption capacity reaches up to around 14.0 mg m^{-2}, which is significantly higher than the calculated *end-on* monolayer coverage value for adsorbed trypsin. The isotherm graph does not show a complete plateau, which indicates the adsorption capacity can also increase further. This reveals, at higher surface density the trypsin molecules get adsorbed either by adjusting in its tertiary conformation or by forming a loosely bound multiple layer on nanoparticle surface. However, formation of multiple layer via weak molecular interaction can also lead to quick desorption of the enzyme molecules from the nanoparticle surface; however we did not observe any detectable trypsin molecules leaking out from the nanoparticle surface while re-dispersing it in enzyme free solution (also described in section 3.2). Hence, we interpret that partial conformational re-arrangement of immobilized trypsin is most likely the principal driving force at higher immobilized surface density.

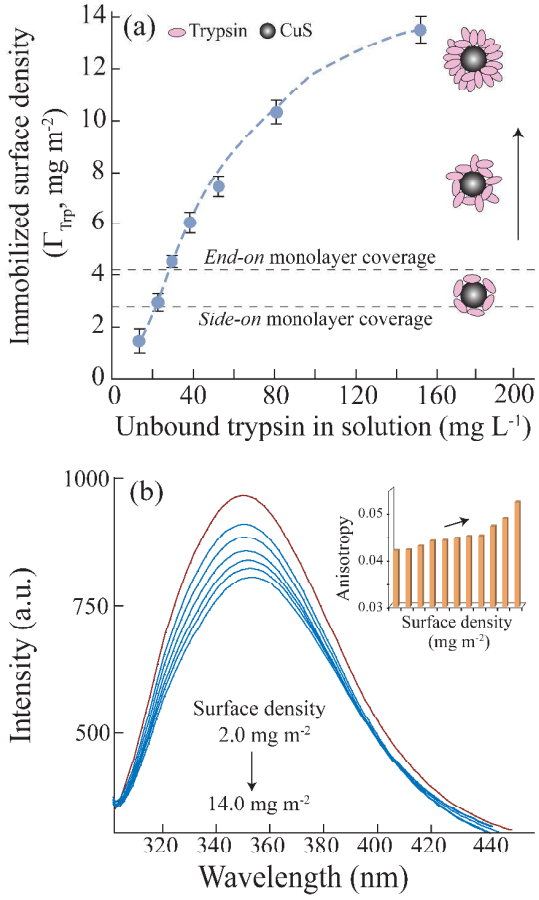


Fig. 5. Adsorption capacity and conformation of trypsin at different surface density: (a) Adsorption isotherm of trypsin on CuS nanoparticles at different initial trypsin concentration. Dashed line fitted through the data points represents the guide to the eye. Two horizontal lines represent the theoretical 'side-on' and 'end-on' monolayer coverage of trypsin on CuS nanoparticles. (b) Steady state fluorescence spectra and (inset) anisotropy values of immobilized trypsin as a function of surface density (2.0 – 14.0 mg m^{-2}) on CuS nanoparticles.

Conformational analysis at different surface density of immobilized trypsin: We have analyzed the in-situ tertiary and secondary conformation of immobilized trypsin at different surface density on CuS nanoparticle surface by steady state fluorescence and FT-IR spectra.

Fluorescence spectra. The enzyme adsorbed nanoparticles were re-dispersed in the enzyme free buffer and further used in the spectroscopic studies for tertiary conformational analysis. The steady state fluorescence emission spectra of adsorbed trypsin as a function of surface density on CuS nanoparticles

are shown in Fig. 5b. The emission intensity of the immobilized trypsin quenched progressively with the increase in its surface density. With the increase in molecular crowding of trypsin on the CuS surface, the available surface area for binding of each trypsin molecules reduces. This could resulted in a partially perturbed tertiary conformation of immobilized trypsin so that more enzyme molecules get accommodated in close proximity on nanoparticle surface, as discussed previously. This intra-molecular and inter-molecular rearrangement of trypsin leads to spatial restriction of the fluorophore in the enzyme molecule, which resulted in partially quenched emission intensity. The anisotropy values of immobilized trypsin molecules are presented (Fig. 5b, inset) after subtracting the anisotropy values of the CuS nanoparticle solution as a background correction. Although the change in anisotropy values with increasing surface density of immobilized trypsin is slighter, it represents an increased spatial restriction of the fluorophore within the enzyme molecule. At higher trypsin surface density ($\geq$10 mg m^{-2}), the increase in anisotropy is more rapid compared to lower surface density; this also indicates a crowding effect starts to play role more significantly on enzyme conformation in this regime. Moreover, considering the aggregation of the enzyme molecules at these concentrations, fluorescence emission spectra of free trypsin solution has also been taken for this whole concentration range. The fluorescence emission spectra of trypsin at different experimental concentration did not show any notable change, from which we assume negligible aggregation of trypsin takes place in this concentration range.

FT-IR spectra. The FT-IR spectra of lyophilized trypsin in native and adsorbed state (with different surface density on CuS nanoparticles) were taken from 4000 to 500 cm^{-1}. The amide I band at 1700−1600 cm^{-1} region is mainly due to the C═O stretching and N−H bending vibration. The band at ~1540−1500 cm^{-1} corresponds to amide II, which represents mainly the C−N stretch vibration (Supporting Information, Fig. S7).[34,35] Although there were no significant peaks of only CuS at amide I region of protein spectrum, the IR spectrum of trypsin immobilized onto CuS surface was analyzed after subtracting the CuS spectrum as a background correction. Further, the individual secondary structural elements of immobilized trypsin at different surface density were quantified by Gaussian deconvolution

analysis of the amide I region (1700−1600 cm^{-1}) of the FT-IR spectra (Supporting Information, Fig. S8).[36,37] The Gaussian distribution peak at 1654 ± 2 cm^{-1} was assigned to α-helix, bands at 1686 ± 1, 1644 ± 1, 1628 ± 1 and 1625 ± 1 cm^{-1} were assigned to β-sheet, and all other peaks were assigned to unordered structural elements including β-turns, random coils, and extended chains.[38] Gaussian analysis reveals that native trypsin has an α-helical content of ~7%, β-sheet of ~34%, and rest ~59% unordered elements (Table 1).

Table 1. Secondary structural content (%) of trypsin in native and different immobilized state.

	Native Trypsin	Surface coverage of adsorbed trypsin (mg m^{-2})				
		2.0	3.8	6.0	10.3	14.0
α-helix (%)	7.3 ± 1	7.2 ± 1	7.0 ± 1	6.7 ± 2	6.6 ± 1	6.1 ± 2
β-sheet (%)	34.2 ± 1	35.8 ± 1	34.8 ± 2	36.3 ± 1	35.9 ± 2	37.1 ± 1
Unorder (%)	58.5 ± 2	57.5 ± 2	58.2 ± 1	57.0 ± 1	57.8 ± 2	57.3 ± 1

Previous studies also reported similar secondary structural content of native trypsin.[39,40] The partial difference in secondary structural content of native trypsin as analyzed by FT-IR spectra and CD spectra (34% β-sheet and 41% β-sheet respectively), can be attributed to the lyophilization induced conformational change in enzyme molecule for FT-IR analysis. The FT-IR data reflects that upon adsorption on CuS surface, the trypsin molecule significantly retain its secondary structural content, throughout all the surface density range studied. Only at higher surface density (> 5 mg m^{-2}), a small loss of α-helical structure of adsorbed trypsin was observed (around 1-2%).

Activity of immobilized trypsin at different surface density: The enzymatic activity of nanoparticle-bound trypsin molecules was measured at different surface density, using BAEE as substrate. The relative specific activity of nanoparticle bound trypsin was measured and presented in percents (%) compared to the same amount of native trypsin activity (Fig. 6a). At a surface density of around 2.0 mg

m^{-2} almost 98% of the native enzyme activity was retained by the adsorbed trypsin molecules (Supporting information, Table S4). Noticeably, in this regime as per theoretical calculation, the immobilized trypsin molecules occupy a complete monolayer coverage on CuS nanoparticle surface. Previously, we have observed a marginal loss of secondary and tertiary structure of immobilized trypsin in this surface density regime (as described in section 3.4.2), which can be related to the high retention of enzymatic activity. At higher surface density of adsorbed trypsin (> 5 mg m^{-2}), partial loss in enzyme activity (around 10-20%) was observed, which can be influenced by the increased alteration in the tertiary conformational of enzyme molecules in this regime. This measured loss in activity of immobilized trypsin can be the consequence of either the decrease in absolute number of active enzyme molecules on CuS surface, or the reduced kinetic parameters of immobilized enzyme-catalysis, or both together. This may indicate towards a heterogeneous distribution in the activity of immobilized enzymes at higher surface density, which however, could not be quantified individually in this study. Hence, we represent the activity loss of immobilized enzyme by measuring the overall product conversion by the total amount of immobilized trypsin after a defined time duration. Within our experimental range, we found at room temperature the activity of immobilized trypsin reduced to ~77% till the surface density reaches to ~14.0 mg m^{-2} on CuS nanoparticles (which is significantly higher than theoretical monolayer coverage value). We attribute this loss of activity to the increased crowding of the enzyme molecules at surface. Further, this data fitted well with a linear equation (Fig. 6a) with a correlation coefficient (R^2) of 0.986, signifying the linear decrease in enzyme activity on CuS nanoparticle decreases with increase in its surface packing density in the studied regime. Fig. 6b represents the direct correlation between the surface density, conformation and in situ enzymatic activity of immobilized trypsin. It is apparent from this graph that, increasing the surface density of immobilized enzyme beyond the monolayer coverage has a simultaneous influence on its in situ activity as well as in its conformation. We have also performed a control experiment by incubating BAEE with bare CuS nanoparticles (without trypsin immobilized), however no detectable amount of hydrolyzed product was observed upon measuring at 253 nm.

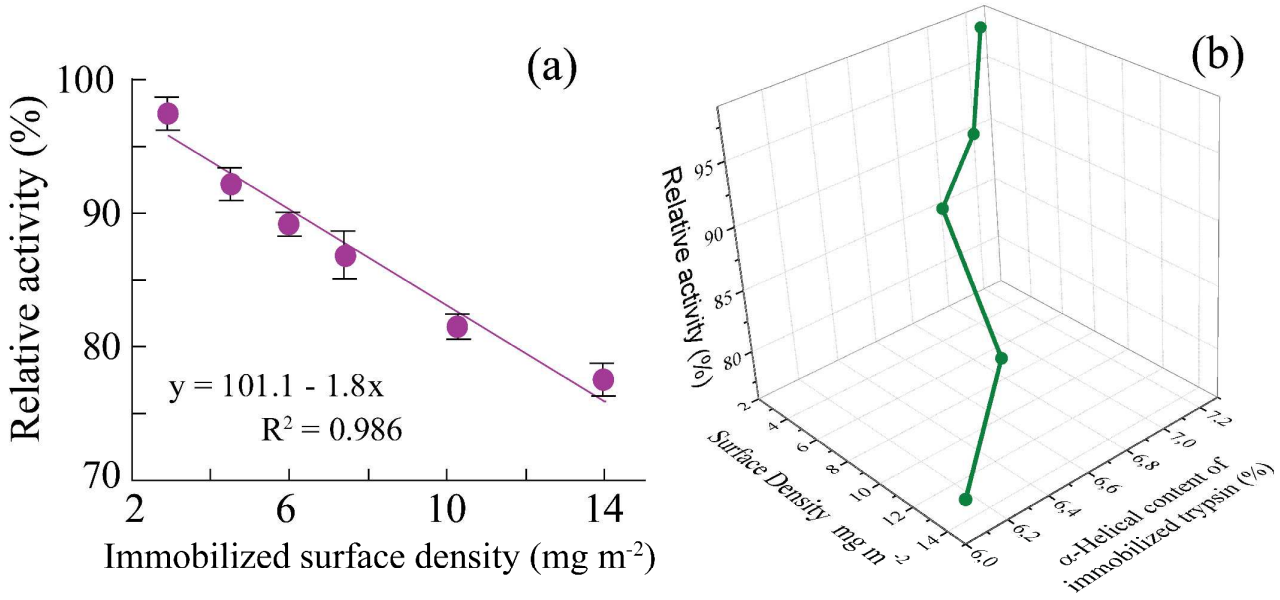


Fig. 6. (a) Retention of enzymatic activity of trypsin molecules as a function of immobilized surface density on CuS nanoparticles. The solid line represents a fit with a linear equation. (b) Direct correlation of surface density (mg m^{-2}), secondary conformation (α-helical content, %) and in situ activity (retention of activity, %) of immobilized trypsin on CuS nanoparticles.

In addition, we have also studied the adsorption capacity, conformation and activity of immobilized trypsin in a temperature range of 10 to 50 ^{o}C, to analyze the stability and functionality of the bio-nanocomposite. Briefly, the adsorption capacity scales down with the increase in temperature from 10 to 40 ^{o}C (from 30.2 to 14.0 mg m^{-2}), while we observed higher enzymatic activity at 40 ^{o}C (~90%). We interpret both the surface density of immobilized enzyme (which also varies at different temperature), and the temperature might have a combine influence on the activity of immobilized trypsin in the temperature range studied here. The conformational analysis using fluorescence spectroscopy and anisotropy reveals partial conformational loss of immobilized trypsin at room temperature (as discussed before), which undergoes further conformational alteration with the increase in temperature. More detailed discussion on temperature influenced interaction of trypsin with CuS nanoparticles is given in the supporting information.

Enzyme kinetics of immobilized trypsin

The enzyme activity kinetics of immobilized trypsin was studied at a surface coverage of 2.0 mg m^{-2} on CuS nanoparticle, and was compared with that of native enzyme molecules. The rate of enzymatic activity, v (mg mL^{-1} min^{-1}) was calculated following the Michaelis-Menten kinetics[41] given by Eq. 2:

$$v = \frac{v_{\max}.[S]}{K_m + [S]} = \frac{k_{cat}.[E]_T.[S]}{K_m + [S]} \quad (2)$$

Where, [S] is substrate (BAEE) concentration (mM), v_{max} is the maximum rate attained at infinite concentration of substrate, and K_m is the Michaelis-Menten constant. The term v_{max} can be further expressed by k_{cat} and $[E]_T$, which is turn over number and total enzyme concentration used for kinetics study, respectively.[41] Fig. 7a shows the Michaelis-Menten plot for the hydrolysis of BAEE by native and immobilized enzyme at pH 8.0 and 25^o C. Maximum kinetic rate (v_{max}) and Michaelis-Menten constant (K_m) values for both free and immobilized enzyme were calculated (Fig. 7b, inset) from the double reciprocal linearized Lineweaver-Burk plot (by re-arranging Eq. 2).

The maximum enzyme kinetic rate (v_{max}) of native and immobilized trypsin was almost similar (0.042 and 0.040 mM min^{-1} respectively). This reveals, the adsorption of trypsin on CuS surface at a low surface density (2.0 mg m^{-2}) has a little influence on the release of the hydrolyzed product of BAEE in the rate limiting step. The Michaelis-Menten constant (K_m) value for native trypsin was marginally decreased upon adsorption on CuS surface (0.35 mM to 0.32 mM), at low surface density (2.0 mg m^{-2}). Although, this change is not highly significant, yet this points towards the increased affinity of the immobilized enzyme for the substrate BAEE. Upon immobilization on nanoparticle surface, the enzyme molecules can interact with the substrate molecules in the solution more rapidly, compared to the diffusion based interaction of native trypsin and substrate molecules in the solution.

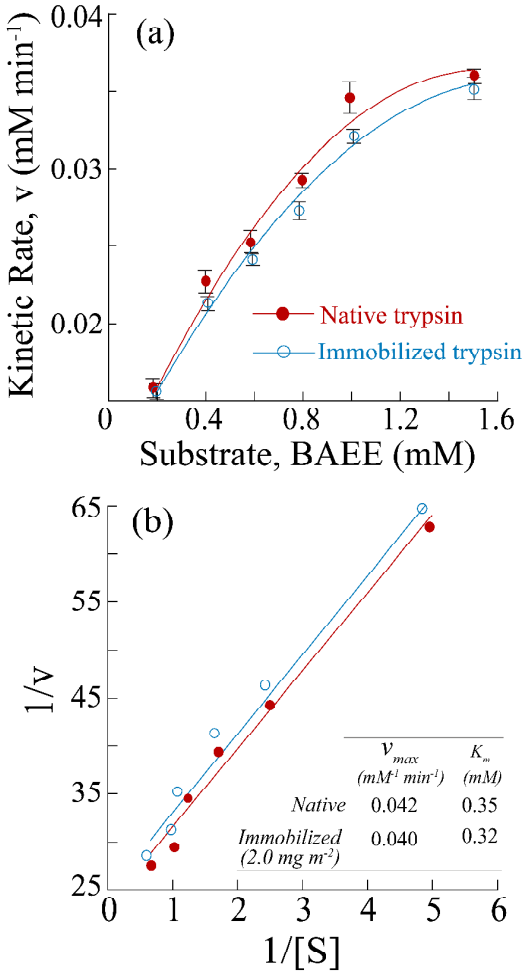


Fig. 7. Enzyme kinetics of native and immobilized trypsin (with 2.0 mg m^{-2} surface density): (a) Michaelis-Menten plot with different BAEE concentration. (b) Double reciprocal Lineweaver-Burk plot of free and immobilized trypsin on CuS nanoparticles. (inset) Enzyme kinetic parameters of free and immobilized trypsin calculated from the Lineweaver-Burk plot.

Conclusion

We have demonstrated the potential use of CuS nanoparticles in developing a high functional bio-nanocomposites using trypsin protease as a model system, with in-depth understanding of structure-functional relationship of the immobilized enzyme. We find an overall stability of the trypsin-CuS nanocomposite in terms of its conformation and activity, at different physico-chemical conditions which can closely resembles various enzymatic assay conditions. Circular dichroism, fluorescence and FT-IR spectroscopy revealed fractional loss of the conformation of trypsin molecules upon binding on CuS

surface. However, immobilized trypsin molecules showed a significant retention of enzymatic activity (>75% to 98%) in the different physico-chemical conditions (immobilized enzyme density, available nanoparticle surface area) at room temperature. We find that density of immobilized trypsin molecules on nanoparticle surface is a dominant parameter which considerably influences its activity, and we have established a linear relationship between the trypsin surface density and its activity. With a monolayer trypsin coverage (~2.0 mg m^{-2}) on CuS surface, the trypsin molecules retains around 98% of its native activity. With further increase in the surface density up to ~14.0 mg m^{-2}, the overall enzymatic activity of immobilized trypsin reduced to around 77%. We attribute this reduction in activity to the steric crowding of trypsin molecules on nanoparticle surface at higher immobilization density (> 5 mg m^{-2}), which overestimated the calculated monolayer coverage. The crowding of enzyme molecules alters the tertiary conformation of immobilized trypsin which impair its overall catalytic activity. The enzyme kinetic analysis of immobilized trypsin with low surface density (~2.0 mg m^{-2}) at room temperature also showed similar kinetic parameters compared to the native enzyme molecules.

The studied trypsin interaction with CuS nanoparticles reveals two significant aspect. First, the implementation of CuS as a potential nano-interface for enzymes in different enzymatic assays, where both the enzyme molecules and CuS nanoparticles can be used as a label free marker to determine the analyte concentration. A simple physisorption of enzyme molecules with the control over the surface density of immobilize enzyme can lead to highly functional bio-nanocomposites. Moreover, high activity of immobilized trypsin can be itself promising for proteomics application. Second, in-depth understanding of the structure-functional relation of trypsin interaction with CuS nanoparticles will facilitate the extrapolation of the crucial parameters which can regulate the enzyme activity on nanoparticle surface. The next step in developing highly functional bio-nanocomposites, would be to investigate different immobilization strategy on CuS nanoparticles and its subsequent activity at defined surface density. Further it would be also scientifically significant to quantify the possible heterogeneity in activity of the immobilized enzyme molecules. This will give a complete image of how different immobilization strategies and physico-chemical conditions lead to different active fraction of

immobilized enzyme, with defined heterogeneity in activity. This knowledge can further be very useful in developing various modern biotechnological applications such as enzymatic assays, diagnostics, drug delivery and therapeutics.

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Electronic Supplementary Information (ESI) Available: Further characterization of CuS nanoparticles (TEM, TGA, EDAX, BET analysis) and interaction with trypsin in terms of kinetics and isotherm; conformational details by FT-IR and CD spectroscopy; influence of temperature on enzyme interaction.

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