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Tuning Enzyme/ α -Zr(IV) Phosphate Nanoplate Interactions via Chemical Modification of Glucose Oxidase

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Abstract

Using glucose oxidase (GOx) and α -Zr(IV) phosphate nanoplates (α -ZrP) as a model system, a generally applicable approach to control enzyme–solid interactions via chemical modification of amino acid side chains of the enzyme is demonstrated. Net charge on GOx was systematically tuned by appending different amounts of polyamine to the protein surface to produce chemically modified GOx(n), where n is the net charge on the enzyme after the modification and ranged from -62 to $+95$ electrostatic units in the system. The binding of GOx(n) with α -ZrP nanosheets was studied by isothermal titration calorimetry (ITC) as well as by surface plasmon resonance (SPR) spectroscopy. Pristine GOx showed no affinity for the α -ZrP nanosheets, but GOx(n) where n -20 showed binding affinities exceeding $(2.1 \pm 0.6) \times 10^6 \text{ M}^{-1}$, resulting from the charge modification of the enzyme. A plot of GOx(n) charge vs Gibbs free energy of binding ($-G$) for $n = +20$ to $n = +65$ indicated an overall increase in favorable interaction between GOx(n) and α -ZrP nanosheets. However, $-G$ is less dependent on the net charge for $n > +45$, as evidenced by the decrease in the slope as charge increased further. All modified enzyme samples and enzyme/ α -ZrP complexes retained a significant amount of folding structure (examined by circular dichroism) as

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.7b02919. Additional data including the agarose gel electrophoresis showing charge ladders of GOx(n) relative to pristine GOx; table of TETA and EDC concentrations used to synthesize specific GOx(n) analogues; GOx(n) circular dichroism spectra and initial activity plots; scheme explaining the method used to determine GOx activity; ITC thermograms of unmodified GOx, GOx(n), and TETA interactions with α -ZrP; SPR sensogram showing reproducibility of α -ZrP surface regeneration; SPR binding kinetic data fitting and EDXS analysis of the GOx(n)/ α -ZrP interaction; circular dichroism spectra and activity plots of GOx(n) after complex formation with α -ZrP; experimental methods for ITC, GOx(n)/ α -ZrP complex formation, binding kinetics of GOx(n)/ α -ZrP, kinetic modeling of GOx(n)/ α -ZrP binding; synthesis and exfoliation of α -ZrP, chemical modification of GOx, agarose gel electrophoresis, GOx activity studies, and circular dichroism studies (PDF)

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Notes

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well as enzymatic activities. Thus, strong control over enzyme–nanosheet interactions via modulating the net charge of enzymes may find potential applications in biosensing and biocatalysis.

Graphical abstract

INTRODUCTION

Elucidating the details of enzyme/nanomaterial interactions could be of fundamental importance for the rational design of functional, stable, and robust enzyme-based sensors,¹ catalysts,² and devices.³ The strong role of electrostatics in the interactions of anionic enzymes and anionic nanosheets was previously hypothesized, and binding has been dramatically improved by flipping the sign of enzyme charge.^{4–6} Chemical control of the electrostatic interactions is vital to modulate affinity for the nanomaterial, activity, stability, and other critical properties of the enzyme. Quantitative binding data in support of the above protein/solid binding hypothesis have been elusive, so far. Using glucose oxidase (GOx) as a model enzyme and α -Zr(IV) phosphate (abbreviated as α -ZrP) as a model nanomaterial, a simple method is reported to control the enzyme/nanomaterial interactions in a predictable manner.

α -ZrP was chosen for the current studies because of its layered structure⁷ which allows it to bind enzymes of a variety of sizes and shapes.⁸ These nanosheets are strongly negatively charged with almost one electrostatic unit of charge per $25 \text{ \AA}^2$, strongly hydrated in aqueous suspensions, and enzymes bound to these nanosheets largely retained their secondary structure and catalytic activities.⁹ The surface area of α -ZrP was estimated to be $100 \text{ m}^2/\text{g}$,¹⁰ thus providing opportunities to achieve high loadings of the enzyme per unit mass.¹¹ For example, proteins^{8,12,13} and peptides¹⁴ have been intercalated into the galleries of α -ZrP. Together, these unique structural characteristics facilitated the study of electrostatic interactions at the enzyme/solid interface. Several useful applications utilizing α -ZrP have been tested including drug delivery,^{15,16} catalysis,¹⁷ ion exchange,¹⁸ and glucose sensing.¹⁹ Furthermore, specific metal ions were successfully used to continuously tune the binding affinity of anionic GOx with the anionic α -ZrP nanosheets,¹¹ revealing that metal ions that have favorable interactions with the enzyme/solid increased the binding affinities the most. For example, GOx/ α -ZrP affinity increased 380-fold in the presence of Zr(IV) and Ca(II) ions, due to their affinities for the phosphate groups of the nanosheets.

The role of enzyme charge on interaction with α -ZrP has been investigated using variously charged proteins and revealed a correlative increase in K_d with protein charge.⁸ Furthermore, the charge of glucose oxidase and hemoglobin has been flipped from negative

to positive to enhance affinities with anionic nanosheets of α -ZrP by orders of magnitude, but increase in affinity as a function of systematic increase in enzyme charge has not been examined.⁴ The design of rational methods to modulate enzyme binding to α -ZrP or other supports in order to enhance performance^{20,21} and enzymatic activities is an unmet challenge.²² Biocatalysis has been the basis of a broad range of applications including the production of amines²³ and synthesis of bionanomaterials,²⁴ and the use of α -ZrP as a nanosupport for such applications is promising.

Glucose oxidase (GOx) was chosen for the current work because its net charge can be tuned over a broad range from -62 (pristine GOx) to very large positive values, as much as +95. The excellent tunability in charge could be useful in identifying strong trends in binding affinities. Binding of glucose oxidase to several solids including polymers,^{25,26} graphene nanocomposites,²⁷ and gold nanoparticle-zinc oxide nanostructures has been examined.²⁸ GOx was tested for applications in biofuel cells^{29,30} and sensors³¹⁻³⁴ and for oxidations using ambient oxygen. For example, GOx catalyzes the oxidation of glucose to gluconic acid while reducing oxygen to hydrogen peroxide.^{35,36} The thermodynamic details of GOx binding to nanosheets and the influence of electrostatic interactions on the affinities are not well understood, but these details are important for practical applications of biocatalysts based on this important enzyme.³⁷

In this work, GOx charge was tuned over a large range and the effect of charge on the thermodynamics and kinetics of GOx binding to ZrP nanosheets has been examined. The COOH groups of GOx were coupled with triethylenetetramine (TETA) by 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC). GOx analogues, indicated as GOx(*n*), where *n* is the net charge on the enzyme after chemical modification, were examined in binding studies with α -ZrP. Our observations are presented below.

EXPERIMENTAL METHODS

Materials

Glucose oxidase from *Aspergillus niger* (GOx, 165 000 MW) was purchased from Calzyme Laboratories, Inc. (Tulelake, CA), and used without further purification.

Triethylenetetramine (TETA) and 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich (St. Louis, MO). Solutions were prepared in 20 mM piperazine-*N,N'*-bis(2-ethane-sulfonic acid) sodium salt (NaPIPES) and 1 mM aqueous tetrabutyl ammonium hydroxide (TBA) (Sigma), pH adjusted to 7.0.

Preparation and Characterization of Exfoliated α -ZrP Thin Films

For SPR experiments, gold SPR sensors (from Reichert catalog no. 13206060) were cleaned with piranha (3:1 H₂SO₄/H₂O₂) solution for 15 min, followed by soaking in ethanol for 20 min and rinsing with excess distilled water. The α -ZrP exfoliated with TBA was spin-cast (Specialty Coating System, model P6700, Indianapolis, IN) at 3000 rpm for 1 min. The α -ZrP coated sensor was dried at room temperature and immersed in reaction buffer overnight for SPR experiments.

Atomic force microscopy (AFM) and scanning electron microscopy (SEM) were used to characterize the α -ZrP films on the sensor chip. Scanning with an Asylum Research MFP-3D AFM (Santa Barbara, CA, USA) was used to assess the integrity of the film. The scans were taken in noncontact mode using Si_3N_4 tips with a force constant of 40 N/m (according to the manufacturer) and a typical resonant frequency of 340 ± 20 kHz. Finally, energy-dispersive X-ray spectroscopy (EDX) analysis of the GOx(*n*) bound α -ZrP SPR sensor was used to measure the nitrogen weight percent after the protein was bound to the sensor surface.

Zeta Potential Measurements

A Brookhaven Zeta Plus zeta potential analyzer (Brookhaven Instrument Corporation, Holtsville, NY) was used for laser Doppler velocimetry studies to measure zeta potential, as described previously.³⁸ In a typical run, the suspensions were prepared using 6 mM α -ZrP and protein concentrations in the range from 40 to 50 μM in 20 mM NaPIPES buffer at pH 7.0. Sample measurements were done in a 4 mL polystyrene cuvette after equilibrating the samples for 30 min at room temperature. The zeta potential values were obtained from electrophoretic mobility of the suspension using a Smoluchowski fit³⁹ using Brookhaven Instrument v 2.3 software provided by the manufacturer.

RESULTS

Chemical Modification of Glucose Oxidase

The net charge on GOx was gradually increased from -62 (pristine GOx at pH 7, pI 4.3)⁴⁰ to $+95$ by reacting the $-\text{COOH}$ residues of the enzyme with increasing amounts of TETA (10–30 mM), under specific conditions of pH, temperature, carbodiimide (EDC) concentration, and reaction time (Scheme 1). Two of the four TETA nitrogens of the modified side chain ($\text{p}K_a$ values = 9.20 and 9.92)⁴¹ are expected to be protonated under these experimental conditions (pH 7). One nitrogen will contribute to the amide bond formation with the COOH group, and the $\text{p}K_a$ value of the fourth nitrogen is too low for protonation to occur. Therefore, a net gain of $+2$ is expected for every TETA appended to GOx, and this modification provided a strong chemical handle to control the charge of GOx(*n*) and provided a simple method to study the influence of enzyme charge on binding to α -ZrP nanosheets at a fixed pH. The resulting GOx(*n*) derivatives have been purified extensively by dialysis to remove unreacted reagents and byproducts, before using them for binding studies. Progress of the chemical modification reaction has been monitored by agarose gel electrophoresis which indicated a charge ladder of GOx(*n*) derivatives (Table S1) with decreasing negative charge or increasing positive charge, as presented below.

Agarose Gel Electrophoresis

The reaction mixture was analyzed by gel electrophoresis to monitor the progress of the chemical modification reaction (Figure 1). Chemically modified GOx-TETA samples were loaded into wells at the center of the gel (white boxes), so that enzyme migration would occur toward the oppositely charged electrode. GOx is strongly negatively charged at pH 7.0 (-62)⁴⁰ and, thus, migrated toward the positive electrode in the agarose gel (Figure 1, lane 1). Modified GOx samples migrated less toward the positive electrode or moved toward the

negative electrode (lanes 3–8) depending on the extent of chemical modification, in a systematic manner. Thus, the net negative charge decreased gradually, until charge reversal occurred (lanes 5–8) where the modified enzyme migrated toward the negative electrode. Variation of TETA and EDC concentrations yielded increasingly charged GOx(*n*) as a charge ladder. Protein modification was carried out by holding the TETA concentration constant (10 mM) and increasing the EDC concentration from 10 to 30 mM (Figure 1, lanes 2–7) in 5 mM increments to yield charge values of −45, +15, +25, +30, +40, and +60 as in lanes 2–7, respectively. Increasing the TETA concentration to 20 mM and the EDC concentration to 60 mM yielded a charge value of +70 (lane 8).

The charge on GOx(*n*) was determined relative to that of pristine GOx, under the same conditions of buffer, pH, and temperature, by measuring the average migration distances in each lane of the above agarose gel. Additional samples were prepared in batches and charges of each sample determined (Figure S1), and the error has been estimated to be ± 5 units. Charge determination was carried out on each batch independently. Samples from batch to batch were examined by agarose gel electrophoresis to determine average migration distances relative to that of the pristine GOx, which are used in turn to determine the net charge on the modified sample. Two key assumptions were made in the charge estimation: (1) enzyme size is unchanged after the chemical modification, so that it did not influence enzyme migration in the gel, and (2) enzyme charge is directly proportional to the distance of its migration under the influence of the applied electric field with no substantial interaction between the enzyme and the agarose matrix. The first assumption is reasonable because the TETA length is negligible when compared to the average diameter of GOx (54 Å), and appending these short chains onto the enzyme surface may not alter its diameter to a significant extent. The validity of the second assumption is reasonable because agarose is charge neutral, but there could be some H-bonding interaction between the amine functions of the TETA chains and the OH groups of the agarose matrix.⁴² Indeed, we suspect that these H-bonding interactions could be responsible for the trailing bands seen in the agarose gels. Therefore, we also tested the estimated charge of one of the samples by mass spectral data, as described below to check our assignments of average charge.

The charge of GOx(+70) assigned by the gel electrophoresis method was also examined by mass spectrometry and *m/z* analysis. A peak shift from 142,896.3 Da observed for pristine GOx to 149,344.3 Da for the modified GOx indicated a total number of 44 TETA side chains per GOx(*n*), and had a net charge of +75.⁴³ Therefore, MS indicated a difference of +5 charge units when compared to electrophoresis methods. Thus, the error associated with the quantification of the number of net charge units by the agarose could be as much as ± 5 units.

Circular Dichroism and Enzyme Activity Studies of GOx(*n*) Derivatives

In order to assess the effect of modification on enzyme structure, circular dichroism (CD) spectroscopy was used. The CD signal (millidegrees) per unit protein concentration (μM) and unit path length (cm) of the cuvette was calculated by appropriate division of the CD signals to obtain the signal as mdeg/ $\mu\text{M}/\text{cm}$. The overall spectral shape of the purified GOx(*n*) sample was comparable to the spectral shape of unmodified GOx, and there has

been substantial retention of the enzyme secondary structure after chemical modification. The far UV CD of GOx, for example, analyzed in 20 mM PIPES at pH 7 with a path length of 0.05 cm and normalized to enzyme concentration, showed peaks corresponding to α -helices (double minima at 210 and 220 nm) and β -sheets at 190 nm (Figure 2A). The CD spectrum of GOx(+45) (red curve) was comparable to that of GOx (blue curve), under the same conditions of path length, buffer, and normalized with respect to enzyme concentration, and all spectra were corrected routinely for any baseline drifts. The molar ellipticities of the modified enzymes (GOx(*n*)) (Figure 2A, inset) were less than those of GOx in the 210–220 nm region. However, the molar ellipticities of GOx(*n*) samples at the 210–220 nm minima were within the range from 87 to 96% of unmodified GOx (Figure S2A), indicating a significant retention of the secondary structure and some loss in the enzyme secondary structure has been noted.

Enzymatic activities of GOx(*n*) were determined and compared with that of the unmodified GOx (Figure 2B) by the standard assay using D-glucose as the substrate (Figure S2B).⁴⁴ GOx catalyzes the oxidation of D-glucose to gluconic acid with concomitant reduction of oxygen to hydrogen peroxide (Figure S3). The enzyme, horseradish peroxidase (HRP), catalyzes the oxidation of guaiacol by hydrogen peroxide to form a colored product (absorption maximum at 470 nm) which is monitored as a function of time (Figures 2B and S2B).

Analysis of the linear portion of the kinetic traces (Figure 2B, red curve) yielded an initial rate that is lower than that of unmodified GOx (blue line) under otherwise the same conditions. Initial rate reductions of GOx(*n*) were in the range from 33 to 60% (Figure S2). These analyses revealed decreasing initial rates with increasing *n*, suggesting that amine conjugation has a negative effect and this could be due to some restriction to the active site or changes in the secondary structure noted in the CD spectra. Next, we examined the binding of representative examples of GOx(*n*) to α -ZrP nanosheets by isothermal titration calorimetry.

Isothermal Titration Calorimetry

The binding of GOx and GOx(*n*) samples (*n* = −20, +25, and +30) to α -ZrP nanosheets was directly monitored by ITC, at 25 °C (20 mM NaPIPES buffer at pH 7.0, Figure 3). Addition of a solution of GOx(*n*) (30–100 μ M) from the automated syringe to an exfoliated suspension of α -ZrP (6 mM, 20 mM NaPIPES buffer at pH 7.0) in the calorimeter resulted in heat release. An exothermic response was observed with a plateau (Figure 3A, blue lines). The dilution curves, representing the heat absorbed when protein solution in the syringe was injected into the calorimeter cell that was filled with buffer solution instead of α -ZrP solution (Figures 3A and S4, red lines), were offset from the titration by 5 μ cal/mol, for clarity. The dilution data were subtracted from the titration data to obtain the corresponding corrected thermograms (Figures 3A and S4, blue lines). The binding of anionic GOx(−20) to the anionic α -ZrP surface was exothermic. The integrated enthalpy data were fitted to a single set of indistinguishable, noncooperative binding sites (Figure 3B, solid curves, eq S1).^{45,46}

Interaction of GOx(+25) (Figure 3B, black curve) or GOx(+30) (blue curve) with α -ZrP also showed binding similar to that of GOx(−20)/ α -ZrP (red curve). The best fit to the data (Figure 3B, Table 1) indicated a H value of -371.3 ± 52.8 kcal/mol, a S value of -1.2 ± 0.2 kcal/mol, a binding constant (K_b) of $(2.1 \pm 0.6) \times 10^6$ M^{−1}, and a G value of -8.6 ± 1.8 kcal/mol for GOx(−20), and similar values were obtained for GOx(+25) and GOx(+30) (Figure 3B, curves have been offset for clarity).

The enthalpy change arising from addition of GOx(n) solution to α -ZrP was plotted as a function of the ratio of concentrations of GOx(n) to α -ZrP (Figure 3B, dotted curves). Enthalpy driven binding was observed here which could be attributed to the improved charge interactions with TETA chains, and these could also contribute to hydrogen bonding interactions with the anionic phosphate groups of the support. Along these lines, direct TETA titration with α -ZrP indicated strong exothermic binding (Figure S4D). On the other hand, titration of α -ZrP with a solution of pristine GOx, under the same conditions as above (Figure S4E) revealed that there has been no detectable heat release/absorption, supporting the hypothesis that GOx chemical modification with TETA facilitated enzyme interaction with the α -ZrP nanosheets. These data are further examined by SPR, which provided real time binding while using very small amounts of the enzyme.

Surface Plasmon Resonance

In a typical SPR experiment, a gold chip is mounted on a prism (Scheme 2) and incident light (laser) on the prism is reflected from the back surface of the gold chip. The amount of light reflected depends on the incident angle, above the critical angle, and the intensity is proportional to the refractive index of the medium adjacent to the surface of the Au layer. A thin α -ZrP layer was coated on the Au surface so that protein binding to the metal phosphate film induces SPR signal. Thus, the chip has been modified to examine protein binding to the metal phosphate in real time by a sensitive and simple method.

An optical detector develops the SPR response vs time signal at different incident angles or at a fixed angle based on the experimental protocol chosen. All our measurements were made at a fixed incident angle to extract the time dependence of the binding event. A solution of GOx(n) (positively charged, red sphere) is flowed over the coating of α -ZrP on the Au sensor chip using a syringe pump that introduces the sample via the inlet. The entire sensor assembly including a dual channel flow cell is mounted on the chip that receives incident light. As the protein binds to the metal phosphate coating, the SPR reflectivity changes in real time and the development of the SPR signal (SPR response vs time) is recorded by the detector.

A thin film of α -ZrP was spin-coated onto the SPR gold chip, and scanning electron micrographs (SEM) (scale bar 50 μ m) of the α -ZrP sensor chip with one half uncoated (Figure 4A) indicated α -ZrP on gold (left) when compared to the bare gold surface (right). An atomic-force microscopy (AFM) image of the α -ZrP coated gold sensor (Figure 4B) showed the topography that indicated α -ZrP nanoplates bound to the surface of the gold chip, and the calculated roughness of this film has been estimated to be 160 nm (± 35 nm). Solutions of (GOx(n)) samples were flowed over the sensor via the sample channel, while the reference channel had the flow of buffer alone with no protein.

A flow rate of 25 $\mu\text{L}/\text{min}$ was used to flow samples at appropriate concentrations of $\text{GOx}(n)$ in 20 mM NaPIPES buffer, pH 7, which indicated good signals with $\alpha\text{-ZrP}$ sensor. As an example, an increase in SPR signal corresponding to the binding of protein to the sensor chip with increasing concentrations of $\text{GOx}(+20)$ (31.25, 62.5, 125, 250, and 500 nM) was observed, reaching equilibrium in about 4–6 min (Figure 4C). At this point, the flow was switched to buffer (without the protein) in both channels to monitor the dissociation kinetics. As this is progressing, a flat line in the reference channel was observed (see Figure S5), which confirms that a flow through of PIPES buffer over the sensor did not interact with $\alpha\text{-ZrP}$ or produce any signal.

After the first run, the SPR chip was regenerated by flowing 0.05% sodium hydroxide (NaOH) for 30 s to remove the bound $\text{GOx}(n)$ and then washed thoroughly with buffer (Figure S5A). The SPR sensor indicated excellent reproducibility after numerous regenerations (Figure S5B). The regeneration of the chip allowed for examining the SPR binding kinetics with a number of $\text{GOx}(n)$ derivatives using the same chip so that there are no chip-to-chip differences. After the chip was thoroughly tested for reproducible binding kinetics, by multiple bindings of the same sample, the binding of other $\text{GOx}(n)$ derivatives with $n = +30, +45, +60$, and $+65$ as well as other control studies (Figure S6) have been examined.

The real time SPR experimental data of $\text{GOx}(n)/\alpha\text{-ZrP}$ onoff kinetic traces were deconvoluted with a 1:1 equilibrium binding model to yield the affinity constants, on-rates, and off-rates. The values of association constant (k_{on}), dissociation rate constant (k_{off}), equilibrium dissociation constant (K_{D}), and binding constant (K_{b}) were collected in Table 2. The change in Gibbs free energy (ΔG) was calculated from the binding constant (K_{b}), and ΔG was plotted as a function of GOx charge (n) (Figure 4D). Initially, for the range of n values between $+20$ and $+45$, $\text{GOx}(n)/\alpha\text{-ZrP}$ affinity became rapidly favorable, as evidenced by the large negative slope. However, $\text{GOx}(n)/\alpha\text{-ZrP}$ affinity was less dependent on charge as n increased from $+45$ to $+65$.

Binding of the proteins to the SPR chip was further confirmed by examining the elemental composition of the sensor energy-dispersive X-ray spectroscopy (EDX) analysis after protein binding. The weight percent values of Zr, P, and N were determined to be 15.57, 9.69, and 2.10 wt %, respectively, indicating the binding of modified $\text{GOx}(+60)$ on the $\alpha\text{-ZrP}$ surface (Figure S6E). There was no significant binding interaction between unmodified GOx and the $\alpha\text{-ZrP}$ nanosheets (Figure S6F), suggesting that binding is facilitated by amines appended to the GOx surface.

Zeta Potential Titrations

If enzyme charge has a significant impact on the affinity for $\alpha\text{-ZrP}$, as demonstrated above, it would be interesting to monitor how the particle charge evolves as the binding proceeds. The change in surface potential was examined by zeta potential titrations of $\alpha\text{-ZrP}$ suspensions in the presence of increasing concentrations of few $\text{GOx}(n)$ derivatives. One hypothesis is that, when $\text{GOx}(n)$ derivatives bind to $\alpha\text{-ZrP}$, the overall surface potential will be affected by four factors: the extent of enzyme binding, the enzyme charge, the initial charge on the nanosheets, and the involvement of ions, if any. If there is no participation of ions in the

binding mechanism, then one might expect nearly neutral charge for the final enzyme covered nanosheets or the binding might stop when the charge is fully neutralized. However, often this is not the case and ions play a major role in controlling the surface potentials of these surfaces.

An increase in net GOx(*n*)/ α -ZrP charge was observed when 3 mM α -ZrP was titrated with increasing concentrations of GOx(*n*) (Figure 5). The zeta measurements are highly reproducible and had no precipitation on the experimental time scale. This could be due to the strong hydrophilic nature of the protein ligand, especially the modified GOx. The zeta measurements were carried out in triplicate, and errors have been statistically acceptable. The net charge increased from -40 to -10 when GOx(-40) was titrated with α -ZrP, providing direct evidence of enzyme binding to the nanosheets. Binding of anionic protein to the anionic solid increased the net charge to around -10 mV (Figure 5). The decrease in charge when a negatively charged protein binds to a negatively charged nanosheet has been explained by counterion participation, discussed later.

Similar plateau values were also attained after the addition of $>100 \mu\text{M}$ GOx(*n*) to α -ZrP suspensions for *n* values of -40 , 0 , $+50$, and $+60$ (gray, green, orange, and blue curves, respectively). The plateau position increased with increasing enzyme charge, which provided another direct evidence for enzyme binding to the nanosheets. This charge evolution may influence the bound enzyme structure, which was examined by CD spectroscopy and activity studies.

Circular Dichroism Studies of GOx(*n*)/ZrP

The secondary structures of GOx(*n*)/ α -ZrP biocatalysts were assessed by CD spectroscopy and compared to the CD spectra of pristine GOx using $1\text{--}3 \mu\text{M}$ protein analyzed in 20 mM PIPES at pH 7. As an example, the spectral shape of the GOx($+45$) bound to α -ZrP (GOx($+45$)/ α -ZrP, Figure 6A, red curve) was comparable to that of unmodified GOx (Figure 6A, blue curve), indicating secondary structure retention of GOx($+45$) after its complex formation with α -ZrP. CD spectra of the α -ZrP suspension alone (Figure S7C) indicated no significant light scattering, and voltage on the detector had values in the range from 550 to 725 V (Figure S7D) within recommended values by the manufacturer. Thus, CD spectra of GOx(*n*)/ α -ZrP were not affected by α -ZrP absorbance or light scattering to a significant extent, under these conditions of low absorbencies at 220 nm and short path lengths (0.05 cm).

The same trend was observed for all GOx(*n*) derivatives when bound to α -ZrP for values of *n* between $+20$ and $+65$ (GOx(*n*)/ α -ZrP, Figure S7A). However, there was some variability in the signal intensity at 220 nm (Figure 6B). While GOx/ α -ZrP (brown bar) and GOx($+65$)/ α -ZrP (green bar) showed comparable intensities to unmodified GOx (blue bar), GOx($+20$)/ α -ZrP (black bar), GOx($+45$)/ α -ZrP (red bar), and GOx($+60$)/ α -ZrP (orange bar) showed reductions at the 220 nm peak minimum by 35, 20, and 27%, respectively, when compared to that of pristine GOx. A comparison of CD values of GOx(*n*)/ α -ZrP with those of the corresponding GOx(*n*) is shown in Figure 6B.

Activities of GOx(*n*)/ α -ZrP Biocatalysts

Initial rates and relative percent specific activities were determined from analysis of the linear portions (first 20 s) of the activity traces, as above, for GOx(*n*)/ α -ZrP samples (Figure S7B). Activities of the bound enzymes (GOx(*n*)/ α -ZrP, Figure 7, even numbered bars) were comparable to activities of the unbound analogues (GOx(*n*), Figure 7, odd numbered bars) under the same conditions of pH, ionic strength, buffer, and temperature. Thus, enzyme/ α -ZrP interaction had a minimal effect on bound enzyme activity, which is remarkable, and a promising result for biocatalysis applications.

However, as discussed earlier, there was some loss in activity due to chemical modification of GOx (34% for GOx(+20) (black bar #3) and 23% for GOx(+45) (red bar #5)). Furthermore, increased modification resulted in more than 50% loss in enzyme activity for GOx(+60) (orange bar #7) and GOx(+65) (green bar #9), suggesting that chemical modification facilitated binding but the extent of modification should be minimized for better activity. Activity decreased from 91% in the case of GOx/ α -ZrP to 77% for GOx(+20)/ α -ZrP and GOx(+45)/ α -ZrP. A decrease in activity to less than 50% was observed for *n* = +60.

DISCUSSION

Chemical modification of the enzyme side chains was used to tune enzyme charge over a very wide range and the results compared with earlier studies.^{4,47–51} Control of charge provided a strong handle to control interactions. Previously,⁴ we have reported modification of GOx with TEPA where the net charge was flipped from net negative to net positive to trigger enzyme binding to anionic nanosheets. However, binding was examined with only one particular charge. Here, charge is continuously tuned over a broad range and binding kinetics/thermodynamics have been quantified for a number of charges. Thus, these enzyme charge ladders provided a powerful method to control the binding interactions between the enzyme and the nanosheets.

Enzyme binding to negatively charged nanosheets was described by the ion-coupled protein-binding model (ICPB).⁴⁵ According to this model, cationic enzymes are surrounded by anionic counterions (Scheme 3).⁵² Binding of positively charged proteins to negatively charged nanosheets would release the counterions from both the enzyme and the nanosheet and contribute to entropy increases (Scheme 3A). On the contrary, the binding of anionic protein (blue sphere) to anionic nanosheets requires sequestration of cations at the interface to offset interfacial charge buildup, at an entropic cost (Scheme 3B). In such cases, the binding is mainly driven by enthalpy loss which overcompensated for the entropy costs.

In support of the ICPB mechanism, protein binding to α -ZrP was influenced by counterion binding/release, protonation/deprotonation, and release of water molecules.⁵³ Here, the ICPB model is applied to test its validity or extension to apply to the current data. For example, the pristine GOx does not bind to α -ZrP nanosheets and this could be due to excessive charge repulsion (Scheme 3B).

In the case of GOx(*n*) derivatives, unfavorable charge repulsion is alleviated to varying extents, and the binding begins to appear as the enzyme charge is increased. Thus, the TETA modification of GOx helped binding to the negatively charged nanosheets due to at least two factors: (1) enhanced favorable charge on the enzyme and (2) direct interaction of the positively charged TETA side chains with the anionic α -ZrP phosphate lattice. Thus, introduction of a certain number of positively charged amine side chains neutralizes enough negative charge on the GOx surface to trigger binding and/or binding is mediated via the direct interaction of TETA chains with the nanosheets.

Binding of the GOx(*n*) derivatives to anionic α -ZrP has been enthalpy driven with an entropic penalty (Table 1). Enthalpy driven binding has been observed when negatively charged proteins like hemoglobin or myoglobin interact with the negatively charged α -ZrP surface,⁵⁴ and entropy driven binding has been observed when positively charged proteins like lysozyme and cytochrome *c* bind α -ZrP.^{45,55} In fact, these studies suggested a linear relationship between the net protein charge and the binding enthalpy.

The large favorable enthalpic changes observed here were nearly equal but opposite in contribution to the unexpected, large unfavorable entropic decreases (Table 1). Above a threshold of charge, changes in G due to GOx(*n*) binding with α -ZrP are small because the S and H terms, as a function of charge, nearly cancel out each other. This kind of balancing has been characterized as enthalpy–entropy compensation and has been widely discussed in enzyme binding to solid surfaces or protein binding to DNA.^{56–58} Furthermore, the large magnitude of enthalpic changes observed in this study (H and S in the range of hundreds of kcal/mol) is probably due to the high affinity of TETA chains for the α -ZrP nanosheets, as observed from the binding data of TETA/ α -ZrP (not shown) where a binding constant in the range of 10^8 M^{-1} was noted. The ITC thermograms are reported for only a few charges where the data could be analyzed with standard binding models. In order to obtain more comprehensive and reproducible data, while using small amounts, SPR experiments were carried out to fully evaluate the binding data with multiple samples.

On the other hand, there is evidence for favorable binding of TETA and TETA derivatives to anionic surfaces. Strongly enthalpic binding was noted when TETA binds to α -ZrP nanosheets (Figure S3C, D). Binding of Hb-TETA derivatives to negatively charged poly(acrylic acid) (PAA) was also enthalpy driven, and there was a correlative increase in H with increasing Hb-TETA charge.⁵⁹ These suggest a meaningful role for TETA at the binding interface, wherein TETA side chains are associated with enthalpy driven binding as in the present studies.

The ICPB mechanism predicts that binding of oppositely charged partners releases counterions from both binding surfaces, since each surface functions as a counterion for the other surface. Binding, under these circumstances, would be under entropy control at an enthalpy penalty of removing counterions from the charged surfaces. Clearly, this is not the case here with $n = +25$ and $+30$ (Table 1), and suggests that additional factors dominate the binding thermodynamics. For example, the direct interaction of the TETA side chains with the phosphate lattice, hydrogen bonding, and protonation of the TETA side chains as they

are brought closer to the anionic nanosheet would also contribute to the binding thermodynamics.

Additionally, further probing of these charge dependent binding interactions between the positively charged GOx and anionic α -ZrP surface was quantitatively determined by surface plasmon resonance spectroscopy. Surface plasmon resonance (SPR) is a powerful label free and ultrasensitive technique to probe the live monitoring of biomolecular interactions occurring in extremely close vicinities of sensors where the environment is very sensitive to refractive index change of the sensing medium.^{60–63} Several previous studies have been used. The various immobilization strategies on the conventional Au sensor include the self-assembled (SAM) molecular monolayers,⁶⁴ various functional polymers,^{65,66} targeted immobilization of bioreceptors,^{61–63} gold nanoparticles, quantum dots,⁶⁷ and other highly refractive graphene based plasmonic materials,^{68,69} which basically improves the sensitivity by means of both surface area and plasmonic properties material. In this study, we spin-coated the α -ZrP onto the Au sensor to get more uniform thin films, which facilitated the more reproducible SPR experiments. α -ZrP is a special inorganic material known to bind both positively and negatively charged proteins with high affinity.

The binding kinetics of the positively charged GOx(n) ranging from $n = +20$ to $n = +65$ with the α -ZrP sensor was studied by the SPR angle or refractive index change after addition of various concentrations and charges of GOx(n) solution to the SPR probing cell (Figure 4C and Figure S6A–D). When the reaction reaches equilibrium, the association and dissociation rates of the protein GOx(n) on the α -ZrP surface are equal. Following the protein injection, the buffer flowed and dissociation of GOx(n) is monitored. The kinetics are sensitive to all stages which include the association phase (i.e., GOx(n) binds to the α -ZrP sensor surface), an equilibrium phase (when on- and off-rates are equal), and the dissociation phase (i.e., buffer rinsing washes away the loosely bound GOx(n) from the α -ZrP sensor surface). All of these kinetic events were quantitatively estimated using a 1:1 equilibrium binding model, and its corresponding kinetic equations are described in the Supporting Information (eqs S2–S4). The individual rate constants for association (k_{on}), dissociation rate constant (k_{off}), equilibrium dissociation (K_D), and binding constants (K_b) were placed in Table 2. The degree of affinity (binding constants (K_b)) was calculated as a ratio of on to off rate constants.

In Table 2, the equilibrium binding constant (K_b) against various GOx(n) charges resulted in a significant increase in affinity toward the α -ZrP solid surface. Unmodified GOx did not show any binding with the α -ZrP solid surface. Furthermore, the SPR binding constants observed here (Table 2) are an order of magnitude higher than those reported for ITC measurements (Table 1). There is a correlative relationship between the value of n (GOx(n) net charge) and the binding constant, since ITC measurements were in the range from $n = -20$ to $n = +30$ and SPR measurements were in the range from $n = +20$ to $n = +65$. Together, these data suggest a minimum number of amines required for GOx(n)/ α -ZrP association and that at a GOx(n) minimum charge of +20 and beyond this threshold a significantly linear relationship is observed between the value of n and the GOx(n)/ α -ZrP binding constant.

Further thermodynamic analysis of SPR binding constants indicates that the GOx/ α -ZrP binding affinity is strongly dependent on electrostatics, as evidenced by the sharp decline in Gibbs free energy (ΔG) as the GOx unit charge is increased over the range from $n = +20$ to $n = +45$ (Figure 4D). The large negative slope in this charge range suggests the dominant role of electrostatics at the GOx/ α -ZrP binding interface due to an increasingly favorable ΔG .

For n values from +45 to +65, ΔG is less dependent on electrostatics, as evidenced by the large reduction in the slope over this charge range. This could be due to an increasing role of H-bonding and van der Waals interactions due to an increasing number of amines on the GOx surface. In fact, these data indicate a threshold number of amines below which there is ΔG charge dependence and above which other factors make a significant contribution to the binding thermodynamics. We recently reported a charge threshold of +30 for the binding of GOx(n) to double helical DNA.⁴³ However, there are many differences between the two, and one is that GOx(n)/ α -ZrP interaction is observed even when $n = -20$. However, there is no observable GOx(n)/DNA interaction below $n = +30$. While the nanosheets are relatively nonflexible, the DNA helix is often considered as a flexible rod. Also, the charge densities per unit area in the case of nanosheets and charge density per unit length of DNA are also expected to play critical roles and may explain the differences in the binding thermodynamics between the two systems.

Zeta potential studies (Figure 5) provided an interesting physical insight into the binding interactions. For example, these revealed that titration of GOx(+50) resulted in a final product with a net surface charge on the GOx(+50)/ α -ZrP being nearly neutral. This implies that the enzyme/solid complex has nearly equal numbers of oppositely charged ions. However, considering the high charge density of α -ZrP (1 charge/25 Å²)¹⁰ and large cross section of GOx (approximated as a sphere with a diameter of 55 Å and area of cross section 2375 Å²), there will be considerable charge differential between the two binding surfaces. To achieve the observed surface charge of ~ 0 in the zeta potential titrations of these samples, sequestration of a large number of cations (up to +50) to the interface between the enzyme and the nanosheet is required. This scenario is consistent with the ICPB mechanism. However, these estimates are rudimentary, as they do not include the contributions of the TETA side chains to the binding and also assume that the entire charge of GOx(n) is present at the proposed contact area.

Circular dichroism findings reported here (Figures S2A and S7A) indicate structure retention of GOx after chemical modification and binding of the modified GOx(n) derivatives to α -ZrP. However, while the CD intensity of the GOx(+65)/ α -ZrP complex was comparable to the intensity of unmodified GOx, there was a reduction in intensities at the 220 nm minimum over the range from 20 to 35%. The key finding is that binding of the GOx(n) to the nanosheets did not introduce any additional loss of structure. This interpretation was supported by the activity data. The enzymatic activity was not affected by interaction of GOx(n) with the nanosheets, indicating potential applications in sensing, catalysis, and device fabrication. However, the observed loss in activity due to TETA modification is in contrast to an increase in activity with TEPA modification. The reason for this difference is unknown at this time. Novel platforms for the immobilization of GOx are currently being explored.⁷⁰ In this study, we have modified GOx to facilitate its binding to

α -ZrP and we have quantified the thermodynamics of binding while GOx activity was unaffected. These data will contribute to the understanding of the underlying mechanisms implicated in GOx immobilization.

CONCLUSIONS

This is the first report of tracking protein binding to the layered solid, α -ZrP, by SPR, to the best of our knowledge, and it provides a real-time means to monitor both thermodynamics as well as kinetics of enzyme binding. The chemical modification of the enzyme resulted in a charge ladder of enzymes where each variant remained enzymatically active, and increased charge increased the binding affinity, initially rather slowly and later more rapidly. For example, SPR data clearly show that the binding affinity improved 2 orders of magnitude when the charge was increased from +20 to +65. Even at the highest affinity, the enzyme remained active and the enzyme/solid complex maintained activity.

Binding of GOx(n) to α -ZrP is an enthalpy driven process in the range from -280 to -430 kcal/mol with an entropic penalty in the range from -0.9 to -1.4 kcal/(mol K). However, binding shows only minor improvement in Gibbs free energy (-9 kcal/mol), initially. This shallow dependence on charge is followed by a more steep increase in binding free energy. The H values observed here are an order of magnitude higher than those reported for Hb-TETA binding to poly(acrylic acid).⁵⁹ This is probably due to the increased amount of surface carboxyls available for conjugation of TETA to GOx when compared to Hb.

SPR studies revealed a strong relationship between GOx(n) charge and the binding constant for values of n between +20 and +65, at which electrostatics dominate the binding. The binding constant increased linearly with n , for $n = +20$ to $n = +65$, and had a large positive slope. Thus, the binding constant can be dialed up as desired, even though this came at a price of enzyme activity. Perhaps, there could be better chemical modification methodologies that would achieve high affinities with less impact on enzyme activity. In addition to electrostatics, there could be additional contributions from H-bonding, ion-dipole interactions, and van der Waals interactions with increasing numbers of TETA chains on the modified GOx. In fact, these data indicate a threshold number of amines below which there is weak G dependence on charge and, above the threshold, the binding thermodynamics are readily controlled by chemical modification. Initial rates of the enzyme activities and enzyme structure after adsorption to the solid are not adversely affected, but there are substantial decreases in specific activities at high charge. Thus, chemical modification needs to be carried out with caution, but it can be used to rationally tune the binding affinities, as demonstrated here. Thus, the chemical modification method tested has potential for controlling the binding interactions with the solid in a predictable manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Wang SG, Zhang Q, Wang R, Yoon SF, Ahn J, Yang DJ, Tian JZ, Li JQ, Zhou Q. Multi-walled carbon nanotubes for the immobilization of enzyme in glucose biosensors. *Electrochem Commun.* 2003; 5:800–803.
2. Phadtare S, Kumar A, Vinod VP, Dash C, Palaskar DV, Rao M, Shukla PG, Sivaram S, Sastry M. Direct assembly of gold nanoparticle “shells” on polyurethane microsphere “cores” and their application as enzyme immobilization templates. *Chem Mater.* 2003; 15:1944–1949.
3. Zimmermann S, Fienbork D, Flounders AW, Liepmann D. In-device enzyme immobilization: wafer-level fabrication of an integrated glucose sensor. *Sens Actuators, B.* 2004; 99:163–173.
4. Chowdhury R, Stromer B, Pokharel B, Kumar CV. Control of Enzyme-Solid Interactions via Chemical Modification. *Langmuir.* 2012; 28(32):11881–11889. [PubMed: 22816873]
5. Song S-W, Hidajat K, Kawi S. Functionalized SBA-15 Materials as Carriers for Controlled Drug Delivery: Influence of Surface Properties on Matrix-Drug Interactions. *Langmuir.* 2005; 21:9568–9575. [PubMed: 16207037]
6. Llopis-Lorente A, Lozano-Torres B, Bernardos A, Martínez-Máñez R, Sancenón F. Mesoporous silica materials for controlled delivery based on enzymes. *J Mater Chem B.* 2017; 5:3069–3083.
7. Clearfield A, Stynes JA. The preparation of crystalline zirconium phosphate and some observations on its ion exchange behavior. *J Inorg Nucl Chem.* 1964; 26:117–129.
8. Deshapriya IK, Kim CS, Novak MJ, Kumar CV. Biofunctionalization of α -zirconium phosphate nanosheets: toward rational control of enzyme loading, affinities, activities and structure retention. *ACS Appl Mater Interfaces.* 2014; 6(12):9643–9653. [PubMed: 24853777]
9. Kumar CV, Chaudhari A. Proteins immobilized at the galleries of layered α -Zirconium phosphate: Structure and activity studies. *J Am Chem Soc.* 2000; 122:830–837.
10. Kim H-N, Keller SW, Mallouk TE, Schmitt J, Decher G. Characterization of zirconium phosphate/polycation thin films grown by sequential adsorption reactions. *Chem Mater.* 1997; 9:1414–1421.
11. Pattammattel A, Deshapriya IK, Chowdhury R, Kumar CV. Metal-enzyme frameworks: Role of metal ions in promoting enzyme self-assembly on α -Zirconium(IV) phosphate nanoplates. *Langmuir.* 2013; 29:2971–2981. [PubMed: 23373444]
12. Bellezza F, Cipiciani A, Costantino U, Negozi ME. Zirconium phosphate and modified zirconium phosphates as supports of lipase. Preparation of the composites and activity of the supported enzyme. *Langmuir.* 2002; 18(23):8737–8742.
13. Díaz A, David A, Pérez R, González ML, Báez A, Wark SE, Zhang P, Clearfield A, Colón JL. Nanoencapsulation of insulin into zirconium phosphate for oral delivery applications. *Biomacromolecules.* 2010; 11(9):2465–2470. [PubMed: 20707305]
14. Xu S, Whittin JC, Yu TT-S, Zhou H, Sun D, Sue HJ, Zou H, Cohen HJ, Zare RN. Capture of phosphopeptides using α -zirconium phosphate nanoplatelets. *Anal Chem.* 2008; 80(14):5542–5549. [PubMed: 18522436]
15. Kalita H, Kumar BP, Konar S, Tantubay S, Mahto MK, Mandal M, Pathak A. Sonochemically synthesized biocompatible zirconium phosphate nanoparticles for pH sensitive drug delivery application. *Mater Sci Eng, C.* 2016; 60:84–91.
16. Díaz A, Saxena V, González J, David A, Casañas B, Carpenter C, Batteas JD, Colón JL, Clearfield A, Hussain MD. Zirconium phosphate nano-platelets: a novel platform for drug delivery in cancer therapy. *Chem Commun.* 2012; 48(12):1754–1756.
17. Kan Y, Clearfield A. Zirconium Phosphate Supported MOF Nanoplatelets. *Inorg Chem.* 2016; 55:5634–5639. [PubMed: 27175935]
18. Silbernagel R, Martin CH, Clearfield A. Zirconium (IV) Phosphonate–Phosphates as Efficient Ion-Exchange Materials. *Inorg Chem.* 2016; 55(4):1651–1656. [PubMed: 26821183]

19. Liu L-M, Wen J, Liu L, He D, Kuang R-y, Shi T. A mediator-free glucose biosensor based on glucose oxidase/chitosan/ α -zirconium phosphate ternary biocomposite. *Anal Biochem.* 2014; 445:24–29. [PubMed: 24135657]
20. Sheldon RA. Enzyme immobilization: the quest for optimum performance. *Adv Synth Catal.* 2007; 349(8-9):1289–1307.
21. Fan Y, Cao X, Hu T, Lin X, Dong H, Zou X. Enhancement of Enzymatic Activity Using Microfabricated Poly (ϵ -caprolactone)/Silica Hybrid Microspheres with Hierarchically Porous Architecture. *J Phys Chem C.* 2016; 120(7):3955–3963.
22. Iwasaki Y, Horiuchi T, Niwa O. Detection of electrochemical enzymatic reactions by surface plasmon resonance measurement. *Anal Chem.* 2001; 73(7):1595–1598. [PubMed: 11321314]
23. Planchestainer M, Contente ML, Cassidy J, Molinari F, Tamborini L, Paradisi F. Continuous flow biocatalysis: production and in-line purification of amines by immobilised transaminase from *Halomonas elongata*. *Green Chem.* 2017; 19(2):372–375.
24. Pavlovic M, Rouster P, Szilagyi I. Synthesis and formulation of functional bionanomaterials with superoxide dismutase activity. *Nanoscale.* 2017; 9(1):369–379. [PubMed: 27924343]
25. Pavlovic M, Rouster P, Szilagyi I. Synthesis and formulation of functional bionanomaterials with superoxide dismutase activity. *Nanoscale.* 2017; 9(1):369–379. [PubMed: 27924343]
26. Malitesta C, Palmisano F, Torsi L, Zambonin PG. Glucose fast-response amperometric sensor based on glucose oxidase immobilized in an electropolymerized poly (o-phenylenediamine) film. *Anal Chem.* 1990; 62(24):2735–2740. [PubMed: 2096737]
27. Zhang Q, Wu S, Zhang L, Lu J, Verproot F, Liu Y, Xing Z, Li J, Song XM. Fabrication of polymeric ionic liquid/graphene nanocomposite for glucose oxidase immobilization and direct electrochemistry. *Biosens Bioelectron.* 2011; 26(5):2632–2637. [PubMed: 21159504]
28. Fang L, Liu B, Liu L, Li Y, Huang K, Zhang Q. Direct electrochemistry of glucose oxidase immobilized on Au nanoparticles-functionalized 3D hierarchically ZnO nanostructures and its application to bioelectrochemical glucose sensor. *Sens Actuators, B.* 2016; 222:1096–1102.
29. Willner I, Katz E, Patolsky F, Bückmann AFJ. Biofuel cell based on glucose oxidase and microperoxidase-11 monolayer-functionalized electrodes. *J Chem Soc Perkin Trans 2.* 1998; 8:1817–1822.
30. Mano N, Mao F, Heller A. A miniature biofuel cell operating in a physiological buffer. *J Am Chem Soc.* 2002; 124:12962–12963. [PubMed: 12405819]
31. Fortier G, Brassard E, Belanger D. Optimization of a polypyrrole glucose oxidase biosensor. *Biosens Bioelectron.* 1990; 5:473–490. [PubMed: 2271147]
32. Gregg BA, Heller A. Cross-linked redox gels containing glucose oxidase for amperometric biosensor applications. *Anal Chem.* 1990; 62:258–263. [PubMed: 2305956]
33. Kang X, Mai Z, Zou X, Cai P, Mo J. A novel glucose biosensor based on immobilization of glucose oxidase in chitosan on a glassy carbon electrode modified with gold-platinum alloy nanoparticles/multiwall carbon nanotubes. *Anal Biochem.* 2007; 369:71. [PubMed: 17678866]
34. Shan C, Yang H, Song J, Han D, Ivaska A, Niu L. Direct electrochemistry of glucose oxidase and biosensing for glucose based on graphene. *Anal Chem.* 2009; 81:2378–2382. [PubMed: 19227979]
35. Gibson QH, Swoboda BEP, Massey V. Kinetics and mechanism of action of glucose oxidase. *J Biol Chem.* 1964; 239:3927–3934. [PubMed: 14257628]
36. Wohlfahrt G, Trivi S, Zeremski J, Peri in D, Leskovac V. The chemical mechanism of action of glucose oxidase from *Aspergillus niger*. *Mol Cell Biochem.* 2004; 260:69–83. [PubMed: 15228088]
37. Wang J. Electrochemical glucose biosensors. *Chem Rev.* 2008; 108:814–825. [PubMed: 18154363]
38. Deshapriya IK, Kumar CV. Nanobio Interfaces: Charge Control of Enzyme/Inorganic Interfaces for Advanced Biocatalysis. *Langmuir.* 2013; 29:14001–14016. [PubMed: 24102555]
39. Scales PJ, Grieser F, Healy TW, White LR, Chan DYC. Electrokinetics of the silica-solution interface: a flat plate streaming potential study. *Langmuir.* 1992; 8:965–974.
40. Pazur JH, Kleppe K. The Oxidation of Glucose and Related Compounds by Glucose Oxidase from *Aspergillus niger*. *Biochemistry.* 1964; 3(4):578–583. [PubMed: 14188176]

41. Nishimura M, Hayashi M, Hayakawa K, Miyazaki M. Ion Chromatographic Study of Sulfate-Complexes in Aqueous Solution. *Anal Sci.* 1994; 10:321.
42. Mehrotra S, Lynam D, Maloney R, Pawelec KM, Tuszynski MH, Lee I, Chan C, Sakamoto J. Time Controlled Protein Release from Layer-by-Layer Assembled Multilayer Functionalized Agarose Hydrogels. *Adv Funct Mater.* 2010; 20(2):247–258. [PubMed: 20200599]
43. Baveghems CL, Pattammattel A, Kumar CV. Designer Histone Complexes: Controlling Protein-DNA Interactions With Protein Charge as ‘All-Or-None’ Digital Switch. *J Phys Chem B.* 2016; 120:11880. [PubMed: 27792341]
44. Sierra JF, Galban J, Castillo JR. Determination of glucose in blood based on the intrinsic fluorescence of glucose oxidase. *Anal Chem.* 1997; 69:1471–1476.
45. Duff MR Jr, Kumar CV. Molecular Signatures of Enzyme-Solid Interactions: Thermodynamics of Protein Binding to α -Zr(IV) Phosphate Nanoplates. *J Phys Chem B.* 2009; 113(45):15083–15089. [PubMed: 19835407]
46. McGhee JD, von Hippel PH. Theoretical aspects of DNA-protein interactions: co-operative and non-co-operative binding of large ligands to a one-dimensional homogeneous lattice. *J Mol Biol.* 1974; 86(2):469–489. [PubMed: 4416620]
47. van Kasteren SI, Kramer HB, Jensen HH, Campbell SJ, Kirkpatrick J, Oldham NJ, Anthony DC, Davis BG. Expanding the diversity of chemical protein modification allows post-translational mimicry. *Nature.* 2007; 446(7139):1105–1109. [PubMed: 17460675]
48. Spicer CD, Davis BG. Selective chemical protein modification. *Nat Commun.* 2014; 5:4740. [PubMed: 25190082]
49. Boutureira O, Bernardes GJ. Advances in chemical protein modification. *Chem Rev.* 2015; 115(5):2174–2195. [PubMed: 25700113]
50. Krall N, da Cruz FP, Boutureira O, Bernardes GJ. Site-selective protein-modification chemistry for basic biology and drug development. *Nat Chem.* 2016; 8(2):103–113. [PubMed: 26791892]
51. Gunnoo SB, Madder A. Chemical Protein Modification through Cysteine. *ChemBioChem.* 2016; 17(7):529–553. [PubMed: 26789551]
52. Duff MR Jr, Kumar CV. Protein-Solid Interactions: Important Role of Solvent, Ions, Temperature, and Buffer in Protein Binding to α -Zr(IV) Phosphate. *Langmuir.* 2009; 25:12635–12643. [PubMed: 19856993]
53. Ding Y, Jones DJ, Maires-Torres P, Roziere J. Two-dimensional nanocomposites: alternating inorganic-organic polymer layers in zirconium phosphate. *Chem Mater.* 1995; 7:562–571.
54. Chaudhari A, Thota J, Kumar C. Binding and cleavage studies of two proteins intercalated at the galleries of α -zirconium phosphate. *Microporous Mesoporous Mater.* 2004; 75(3):281–291.
55. Duff MR Jr, Kumar CV. Protein-Solid Interactions: Important Role of Solvent, Ions, Temperature, and Buffer in Protein Binding to α -Zr(IV) Phosphate. *Langmuir.* 2009; 25(21):12635–12643. [PubMed: 19856993]
56. Lumry R, Rajender S. Enthalpy-Entropy compensation phenomena in water solutions of proteins and small molecules: a ubiquitous property of water. *Biopolymers.* 1970; 9:1125–1227. [PubMed: 4918636]
57. Liu L, Yang C, Guo Q-X. A study on the enthalpy-entropy compensation in protein unfolding. *Biophys Chem.* 2000; 84:239–251. [PubMed: 10852311]
58. Tzeng S-R, Kalodimos CG. Dynamic activation of an allosteric regulatory protein. *Nature.* 2009; 462:368–372. [PubMed: 19924217]
59. Thilakarathne VK, Briand VA, Kasi RM, Kumar CV. Tuning Hemoglobin-Poly (acrylic acid) Interactions by Controlled Chemical Modification with Triethylenetetramine. *J Phys Chem B.* 2012; 116(42):12783–12792. [PubMed: 23030246]
60. Homola J, Yee SS, Gauglitz G. Surface plasmon resonance sensors: review. *Sens Actuators, B.* 1999; 54:3–15.
61. Chang CC, Chiu NF, Lin DS, Chu-Su Y, Liang YH, Lin CW. High-sensitivity detection of carbohydrate antigen 15–3 using a gold/zinc oxide thin film surface plasmon resonance-based biosensor. *Anal Chem.* 2010; 82:1207–1212. [PubMed: 20102177]
62. Nguyen B, Tanious FA, Wilson WD. Biosensor-surface plasmon resonance: quantitative analysis of small molecule-nucleic acid interactions. *Methods.* 2007; 42:150–161. [PubMed: 17472897]

63. Di Primo C, Dausse E, Toulme JJ. Surface plasmon resonance investigation of RNA aptamer-RNA ligand interactions. *Methods Mol Biol.* 2011; 764:279–300. [PubMed: 21748648]
64. Chaki NK, Vijayamohanan K. Self-assembled monolayers as a tunable platform for biosensor applications. *Biosens Bioelectron.* 2002; 17:1–12. [PubMed: 11742729]
65. Briand VA, Thilakarathne V, Kasi RM, Kumar CV. Novel surface plasmon resonance sensor for the detection of heme at biological levels via highly selective recognition by apo-hemoglobin. *Talanta.* 2012; 99:113–118. [PubMed: 22967529]
66. Kamat RK, Zhang Y, Anuganti M, Ma W, Noshadi I, Fu H, Ekatan S, Parnas R, Wang C, Kumar CV, Lin Y. Enzymatic Activities of Polycatalytic Complexes with Nonprocessive Cellulases Immobilized on the Surface of Magnetic Nanoparticles. *Langmuir.* 2016; 32:11573–11579. [PubMed: 27797206]
67. Malic L, Sandros MG, Tabrizian M. Designed biointerface using near-infrared quantum dots for ultrasensitive surface plasmon resonance imaging biosensors. *Anal Chem.* 2011; 83:5222–5229. [PubMed: 21604742]
68. Szunerits S, Maalouli N, Wijaya E, Vilcot JP, Boukherroub R. Recent advances in the development of graphene-based surface plasmon resonance (SPR) interfaces. *Anal Bioanal Chem.* 2013; 405:1435–1443. [PubMed: 23314618]
69. Pattammattel A, Kumar CV. Kitchen Chemistry 101: Multigram Production of High Quality Biographene in a Blender with Edible Proteins. *Adv Funct Mater.* 2015; 25:7088–7098.
70. Haghighi N, Hallaj R, Salimi A. Immobilization of glucose oxidase onto a novel platform based on modified TiO₂ and graphene oxide, direct electrochemistry, catalytic and photocatalytic activity. *Mater Sci Eng, C.* 2017; 73:417–424.

Figure 1.

Agarose gel of GOx and GOx(*n*) conjugates at pH 7.0 in 40 mM Tris–acetate. Samples were spotted at the center of the gel, and they migrated to the opposite electrode. Net charges estimated from the electrophoretic mobilities of samples in each lane are shown at the bottom.

Figure 2.

(A) Circular dichroism spectra and (B) initial enzymatic activities of GOx(+45) (red curves) compared to those of GOx (blue curves). The initial activity of GOx(+45) (red line) showed reduction in initial rate by 23% when compared to that of GOx.

Figure 3.

Isothermal titration calorimetry data for the titration $\text{GOx}(n)$ into $\alpha\text{-ZrP}$, in 20 mM NaPIPES buffer at pH 7.0. (A) The thermogram obtained by the addition of $61 \mu\text{M}$ $\text{GOx}(-20)$ solution to 6 mM $\alpha\text{-ZrP}$ (blue curve) and the dilution data (red curve) which is offset from the titration data for clarity. (B) Integrated heat as a function of the molar ratio of $\text{GOx}(-20)$, $\text{GOx}(+25)$, or $\text{GOx}(+30)$ to $\alpha\text{-ZrP}$. The red, black, and blue lines are best fits to the $[\text{GOx}(-20)]/[\text{ZrP}]$, $[\text{GOx}(+25)]/[\text{ZrP}]$, and $[\text{GOx}(+30)]/[\text{ZrP}]$ data sets, respectively.

Figure 4.

(A) Scanning electron micrograph (scale bar 50 μm) of α -ZrP coated on a Au sensor (left half of the SEM image) in contrast to an uncoated Au sensor (right half of the SEM image). (B) AFM scan of α -ZrP coating on the sensor chip with an estimated roughness of 160.0 nm (± 35). (C) Association kinetics (0–350 s) of GOx(+20) with the α -ZrP sensor at 25 $\mu\text{L min}^{-1}$ (black bubbles are experimental data; the solid red line is the fit) of GOx(+20) with increasing concentrations. (D) Correlation of GOx(n) charge with binding free energy (ΔG) deduced from the binding constant (K_b) obtained by SPR.

Figure 5.

Zeta potential titration of α -ZrP (3 mM) with GOx(n) at 25 °C in 20 mM PIPES buffer, pH 7.0. GOx charge (n) was varied from -40 to +60 as marked. In each case, the charge gradually increased from -40 and reached a plateau which was not necessarily at zero charge.

Figure 6.

Circular dichroism spectra of $\text{GOx}(n)/\alpha\text{-ZrP}$ when compared to GOx: (A) Circular dichroism spectra of GOx (blue) and $\text{GOx}(+45)/\text{ZrP}$ (black). GOx α -helical structure is retained after GOx conjugation to TETA and complex formation with α -ZrP to yield $\text{GOx}(+45)/\alpha\text{-ZrP}$. (B) Ellipticities of GOx (blue bar), $\text{GOx}/\alpha\text{-ZrP}$ (brown bar), $\text{GOx}(+65)/\alpha\text{-ZrP}$ (green bar), $\text{GOx}(+20)/\alpha\text{-ZrP}$ (black bar), $\text{GOx}(+45)/\alpha\text{-ZrP}$ (red bar), and $\text{GOx}(+60)/\alpha\text{-ZrP}$ (orange bar).

Figure 7. Relative specific activities of $\text{GOx}(n)/\alpha\text{-ZrP}$ and $\text{GOx}(n)$, as marked, under the same conditions of buffer, pH, and temperature. These activities of $\text{GOx}(n)$ derivatives were calculated as a percentage of the specific activity of unmodified GOx .

Scheme 1. GOx(*n*) Binding to Exfoliated α -ZrP Nanosheets^a

^aThe COOH groups of GOx, amidated with increasing numbers of TETA using carbodiimide chemistry, resulted in tuning of GOx surface charge (*n*) from negative (pristine GOx, dark blue sphere) to positive (red sphere). The negatively charged GOx (−62) does not bind to the negatively charged α -ZrP plates, but charge reversal from negative to positive (light blue, yellow, and red spheres) via chemical modification triggered GOx binding to the nanosheets. A schematic structure of α -ZrP³⁸ (inset) is shown.

Scheme 2. Surface Plasmon Resonance (SPR) Experimental Setup Showing Protein Binding to a Au Chip Coated with a Thin Layer of α -ZrP^a

^aThe expected kinetic plot as the protein binds to the sensor surface and AFM image of the sensor chip coated with α -ZrP are also shown.

Scheme 3. Ion Coupled Protein-Binding (ICPB) Model for Enzyme Binding to α -ZrP Nanosheets

Table 1

ITC Data for the Binding of GOx(n) to α -ZrP at 25 °C in 20 mM NaPIPES at pH 7.0 (Averages of Three Separate Measurements)

GOx(n) charge (± 5)	$K_b \times 10^6$ (M ⁻¹)	$T \ S$ (kcal/mol)	H (kcal/mol)	G (kcal/mol)
GOx (pristine)	0	0	0	0
-20	2.1 ± 0.6	-357.6 ± 59.6	-371.3 ± 52.8	-8.6 ± 1.8
+25	3.8 ± 2.8	-417.2 ± 29.8	-427.2 ± 39.0	-9.0 ± 2.8
+30	3.4 ± 1.1	-268.2 ± 59.6	-277 ± 57.4	-8.9 ± 2.5

Table 2SPR Determined Kinetic Parameters for GOx(n) Binding to α -ZrP

GOx charge (n)	$k_{\text{on}} \times 10^3$ ($\text{M}^{-1} \text{s}^{-1}$)	$k_{\text{off}} \times 10^{-4}$ (s^{-1})	$K_{\text{D}} \times 10^{-8}$ (M)	$K_{\text{b}} \times 10^7$ (M^{-1})
+20	2.1 (± 0.1)	27.5 (± 0.4)	133 (± 9.0)	0.075 (± 0.1)
+30	5.1 (± 0.1)	5.7 (± 0.2)	11.3 (± 0.5)	0.88 (± 0.1)
+45	8.8 (± 0.1)	2.7 (± 0.2)	3.0 (± 0.2)	3.3 (± 0.2)
+60	11.9 (± 0.1)	2.5 (± 0.0)	2.1 (± 0.2)	4.8 (± 0.0)
+65	9.0 (± 0.1)	1.6 (± 0.2)	1.8 (± 0.2)	5.6 (± 0.2)