2017

Enhanced enzymatic activity from phosphotriesterase trimer gold nanoparticle bioconjugates for pesticide detection

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Abstract
The rapid detection of organophosphates (OPs), a class of strong neurotoxins, is critically important for monitoring acute insecticide exposure and potential chemical warfare agent use. Herein, we improve the enzymatic activity of a phosphotriesterase trimer (PTE3), an enzyme that selectively recognizes OPs directly, by conjugation with distinctly sized (i.e., 5, 10, and 20 nm diameter) gold nanoparticles (AuNPs). The number of enzymes immobilized on the AuNP was controlled by conjugating increasing molar ratios of PTE3 onto the AuNP surface via metal affinity coordination. This occurs between the PTE3-His6 termini and the AuNP-displayed Ni2+-nitrilotriacetic acid end groups and was confirmed with gel electrophoresis. The enzymatic efficiency of the resultant PTE3–AuNP bioconjugates was analyzed via enzyme progress curves acquired from two distinct assay formats that compared free unbound PTE3 with the following PTE3–AuNP bioconjugates: (1) fixed concentration of AuNPs while increasing the bioconjugate molar ratio of PTE3 displayed around the AuNP and (2) fixed concentration of PTE3 while increasing the bioconjugate molar ratio of PTE3–AuNP by decreasing the AuNP concentration. Both assay formats monitored the absorbance of p-nitrophenol that was produced as PTE3 hydrolyzed the substrate paraoxon, a commercial insecticide and OP nerve agent simulant. Results demonstrate a general equivalent trend between the two formats. For all experiments, a maximum enzymatic velocity (Vmax) increased by 17-fold over free enzyme for the lowest PTE3–AuNP ratio and the largest AuNP (i.e., ratio of 1[thin space (1/6-em)]:[thin space (1/6-em)]1, 20 nm dia. AuNP). This work provides a route to improve enzymatic OP detection strategies with enzyme–NP bioconjugates.

Disciplines
Applied Mechanics | Environmental Indicators and Impact Assessment | Mechanical Engineering | Nanoscience and Nanotechnology

Comments

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Enhanced enzymatic activity from phosphotriesterase trimer gold nanoparticle bioconjugates for pesticide detection†

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The rapid detection of organophosphates (OPs), a class of strong neurotoxins, is critically important for monitoring acute insecticide exposure and potential chemical warfare agent use. Herein, we improve the enzymatic activity of a phosphotriesterase trimer (PTE3), an enzyme that selectively recognizes OPs directly, by conjugation with distinctly sized (i.e., 5, 10, and 20 nm diameter) gold nanoparticles (AuNPs). The number of enzymes immobilized on the AuNP was controlled by conjugating increasing molar ratios of PTE3 onto the AuNP surface via metal affinity coordination. This occurs between the PTE3-His6 termini and the AuNP-displayed Ni2+–nitrilotriacetic acid end groups and was confirmed with gel electrophoresis. The enzymatic efficiency of the resultant PTE3–AuNP bioconjugates was analyzed via enzyme progress curves acquired from two distinct assay formats that compared free unbound PTE3 with the following PTE3–AuNP bioconjugates: (1) fixed concentration of AuNPs while increasing the bioconjugate molar ratio of PTE3 displayed around the AuNP and (2) fixed concentration of PTE3 while increasing the bioconjugate molar ratio of PTE3–AuNP by decreasing the AuNP concentration. Both assay formats monitored the absorbance of p-nitrophenol that was produced as PTE3 hydrolyzed the substrate paraoxon, a commercial insecticide and OP nerve agent simulant. Results demonstrate a general equivalent trend between the two formats. For all experiments, a maximum enzymatic velocity (Vmax) increased by 17-fold over free enzyme for the lowest PTE3–AuNP ratio and the largest AuNP (i.e., ratio of 1:1, 20 nm dia. AuNP). This work provides a route to improve enzymatic OP detection strategies with enzyme–NP bioconjugates.

Introduction

Organophosphates (OPs) are widely used as neuroinhibitory pesticides to prevent crop loss.1 However, OPs pose a significant risk to the environment through water run-off and an even greater risk to humans in the form of nerve agents that are used in chemical weapons (e.g., Soman and Sarin).2 OPs released into the environment degrade with time, but such degradation has not prevented trace levels appearing in drinking water supplies.3 Therefore, there is a strong need to produce sensors that can rapidly assess and continuously monitor exposure/contamination from OP-based pesticides and chemical warfare/terror agents before such chemicals have the opportunity to leech deep into the soil. Enzyme-based biosensors display tremendous promise for rapid, continuous, and low-cost biosensing needed for in-field OP sensing.4–8 Several enzymes have been explored for enzymatic OP sensing including organophosphate hydrolase (OPH).9 OPH is a lipoprotein that hydrolyzes the triester bond of OP pesticides10 such as paraoxon, methyl-parathion and diazinon11 and has been shown to hydrolyze paraoxon rapidly – paraoxon breakdown by OPH appears to approach the diffusion limit of the substrate (V/KM ≈ 106–109 M−1 s−1).12 Phosphotriesterase from Brevundimonas diminuta is an OPH variant of specific interest as it is able to selectively target triple O-linked phosphonate centers such as those found in Sarin and Tabun.13 Multiple derivative OPH structures, including a de novo
chimeric collagen-PTE trimer (PTE$_3$), have been engineered to increase the enzyme’s activity within specific and targeted applications.$^{14}$ However, the reaction kinetics and stability of OPH and some of its derivatives are currently not considered sufficiently robust nor sufficiently sensitive for use in portable, field-deployable OP biosensing devices.

For decades, researchers have been exploring possible solutions for increasing the stability and activity of OPH with diverse immobilization techniques. For example, protein fusion, which anchors OPH onto the surface of Escherichia coli using an Lpp-OmpA fusion protein, demonstrated a seven-fold increase in parathion degradation.$^{15}$ OPH entrapment within a polyurethane foam matrix provided increased enzymatic life (1.8 days soluble and 278 days immobilized when stored at 25 °C) and thermal stability (1.5 hours soluble and 158.4 hours immobilized at 50 °C).$^{16}$ Researchers have also demonstrated that OPH retained enhanced enzymatic stability when attached to nanomaterials such as silica matrices (no significant loss after 12 months),$^{17}$ amyloid fibrils (300% increase in relative temperature stability at 40, 45, and 50 °C),$^{18}$ carbon nanotubes (only 25% signal loss after 7 months),$^{19}$ and nanoparticles (NPs) such as nanocrystalline semiconductor quantum dots (QDs).$^{20}$ The latter provide bio-compatible surfaces, potential for enhanced conductivity through direct enzyme electrical ‘wiring’,$^{21}$ and high surface area for immobilization of enzymes. In this vein, we recently demonstrated that PTE$_3$ has enhanced activity when displayed on CdSe/ZnS core/shell QDs.$^{20}$ Indeed, recent research has shown that enzyme immobilization on NPs in general can increase both the sensitivity and stability of enzymes for a variety of applications.$^{22-24}$

While the exact interaction and catalytic nature between NPs and enzymes are complex and much is still unknown, the specific characteristics of enzyme–NP conjugates such as mass transport, enzyme orientation, surface morphology, and enzyme density have been shown in many cases to enhance enzymatic performance.$^{24-26}$ NPs have unique properties of high surface-to-volume ratios as well as high radii of curvature which can potentially allow enzymes to be positioned with increased distance between adjacent immobilized enzymes; this may limit unfavorable protein to protein interaction(s) on the NP surface.$^{27}$ Enzyme–NP bioconjugates can be further manipulated by changing the attachment chemistry of the enzyme in order to situate the enzyme’s binding pocket away from the NP while optimizing the position for substrate to diffuse to and from the enzyme. Enzymes that have been immobilized onto the surface of NPs display improvements in activity along with wider performance window across a range of pH and temperature changes.$^{28}$ These advantages have not only provided enhanced enzyme activity, stability, and specificity, but have also improved the ability of enzymes to be used in sensors for a variety of conditions such as for the highly sensitive detection of glutamate, glucose, lactate in simulated or actual biological solutions/conditions.$^{28-33}$ Moreover, we have shown that immobilizing the enzyme alkaline phosphatase onto QDs can improve the enzymatic efficiency ($k_{cat}/K_M$) up to 40% versus free enzyme.$^{23}$ We have also shown that immobilizing trypsin substrate onto NPs can enhance enzymatic activity in Förster resonance energy transfer (FRET) assays where the rate of trypsin-catalyzed proteolysis of QD-displayed peptide increased to five times that of free enzyme.$^{34}$ AuNPs have shown enhanced effects on a variety of small molecules such as improved affinity for Alzheimer’s peptide Aβ (a factor of 7 higher for NP-immobilized D3 than for the free ligands)$^{35}$ and anti-viral lectin Cyanovirin-N (several orders of magnitude higher than isolated monomeric sugars interacting with the lectin),$^{36}$ increased enzyme selectivity of α-chymotrypsin (∼3-fold improvement for cationic substrates),$^{37}$ and lowered $K_M$ values of glucose oxidase (1.56-fold lower in magnitude than free glucose oxidase)$^{38}$ however, the enhanced enzymatic performance of OPH using AuNP has not been researched. Here, we improve the performance of PTE$_3$ via immobilization onto gold nanoparticles (AuNPs). PTE$_3$ is attached to the AuNP via metal-affinity interactions allowing for ratiometric and orientation control on the AuNP.$^{39}$ This method orients the catalytic site on the outside of the bioconjugate increasing the likelihood of enzyme substrate binding to occur.$^{40}$ PTE$_3$ was immobilized onto 5, 10, and 20 nm diameter AuNPs in an effort to analyze trends between enzyme activity and NP carrier size/curvature along with immobilized enzyme density, see Fig. 1. In this work, the organophosphate paraoxon was used as substrate since its hydrolysis produces $p$-nitrophenol which has a distinct, measurable absorbance (405 nm).$^{14}$ PTE$_3$–AuNP performance was monitored in two distinct

**Fig. 1** Left: Schematic diagram of AuNP–PTE$_3$ conjugate preparation. AuNPs were surface functionalized with 50% dihydrolipoic acid (DHLA) and 50% nitritriacetic acid appended dihydrolipoic acid (DHLA–NTA). The NTA groups were preloaded with Ni$^{2+}$ as described in the Methods. PTE$_3$ coordinates by metal affinity to the Ni$^{2+}$–NTA displayed around the AuNPs to yield the final AuNP–PTE$_3$ bioconjugate. Right: Hydrolysis of paraoxon due to OPH catalyst into $p$-nitrophenol. Note, figure is not drawn to scale.
formats: fixed amounts of AuNPs where the molar ratio of PTE₃ per AuNP was systematically increased and fixed concentrations of PTE₃ where the amount of AuNPs were systematically decreased to increase the ratio of PTE₃/AuNP. The data again confirms enzymatic enhancement of PTE₃ when displayed on NP and that this can be extended to other types of NP materials with different sizes utilizing different enzyme attachment chemistries.

Experimental methods

Chemicals

Paraoxon was purchased from Chem Services (USA). All other chemicals including solvents were purchased from Sigma-Aldrich (USA) or Acros Organics (USA), unless indicated otherwise, and used as received without any other further purification unless stated. The use of these chemicals are outlined in the following sections of this Experimental methods section.

PTE₃ expression

Briefly, the gene cassette encoding the phosphotriesterase gene (PTE, EC 3.1.8.1) from *Brevundimonas diminuta* and the collagen-like protein from *Streptococcus pyogenes* was synthesized by Genescr ipt. The multimerization domain consisted of the V-domain which facilitates assembly of the structure and a collagen helix comprised of 78 repeats of the glycine trimer (Gly-Xaa-Yaa). The cassette was flanked by unique, terminal restriction enzyme cleavage sites which facilitated cloning into either the cytoplasmic or periplasmic pET bacterial expression plasmids (Takara Bio, USA). Integration into either expression construct allowed for the addition of a C-terminal hexahistidine tag (His₆) that is used for purification of the protein with immobilized metal affinity chromatography (IMAC). Additional details on vector construction can be found in our previous work.¹⁴

Expression of the PTE₃ was performed in *E. coli* strain BL21 (DE3). Briefly, 500 mL of Terrific Broth containing the appropriate antibiotic was inoculated with 5 mL of an overnight culture. The culture was maintained at 37 °C for 3 hours or until mid-log phase was reached. Expression of the recombinant protein was induced with isopropyl-ß-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.25 mM. The culture was maintained at 30 °C for 15–24 hours then centrifuged at 4000g to pellet the cells. The resultant cell pellet was frozen at −80 °C for a minimum of 3 hours to aid in cell lysis. Following the incubation at −80 °C, cells were resuspended in 30–50 mL of lysis buffer (0.5× phosphate buffered saline – PBS, 69 mM NaCl; 1.4 mM KCl, pH 7.4), 1 mM ethylenediamine tetraacetic acid (EDTA), 0.1% Triton X-100, 2 mg mL⁻¹ lysozyme and incubated on ice for 30 minutes with constant, slow agitation. The cell slurry was then sonicated 6 times at 30 seconds intervals using a Branson Sonifier (constant output, duty cycle 5). Soluble and insoluble material was separated via centrifugation at 15 000g for 30 minutes. Soluble material was decanted to a fresh conical tube and combined with Ni²⁺-nitrilotriacetic acid (Ni-NTA) resin (GE Healthcare). The insoluble material was reserved for later analysis via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein was immobilized to the IMAC resin via batch mixing for 3 hours at 4 °C. Following this incubation, the resin was batch washed with 30–40 bed volumes of wash buffer (20 mM phosphate pH 7.5, 150 mM NaCl, 25 mM imidazole). Protein was eluted from the column using wash buffer with imidazole at a final concentration of 200 mM. Formation of the triple helix was not spontaneous. IMAC purified protein was incubated for an additional 48–72 hours to allow for formation of the multimer. Size exclusion chromatography was performed using an Enrich SEC650 fast protein liquid chromatography (FPLC) column and BioRad Biologic System to separate the triple helix construct from the monomeric protein. Additional details regarding these processes can be found in previous work.¹⁴,²³

Gold NP synthesis and preparation

Citrate-modified AuNPs were first synthesized using a slightly modified citrate reduction protocol and the final NTA-modified AuNPs were obtained using a second ligand exchange method as described elsewhere.¹⁴,²³ For the AuNP synthesis, 100 mM of HAuCl₃·3H₂O stock solution and 200 mM of trisodium citrate (Na₃C₆H₅O₇) were prepared in deionized water. Next, 100 µL of stock HAuCl₃·3H₂O was added to 50 mL of water and vigorously stirred for 1 min followed by the addition of 200 µL or 50 µL citrate stock solution for the creation of the 5 nm and 10 nm AuNPs, respectively. After 5 min of stirring, 100 µL of 100 mM NaBH₄, freshly prepared in water, was added to the reaction solution and stirred for an additional 20 min for the reduction and formation of gold colloids. The 20 nm citrate-modified AuNPs were prepared in 50 µL of citrate stock solution in a similar manner except the solution was boiled for 30 min without NaBH₄.

For preparing NTA-modified AuNPs, 10 mM of as-synthesized citrate-modified AuNPs were mixed with excess amount of mixed ligand stock solution (100, 50, 25 µL of 100 mM stock for the 5, 10, 20 nm AuNPs, respectively) containing 50% of dihydrolipoic acid (DHLA) and 50% NTA-appended dihydrolipoic acid (DHLA-NTA) which had been deprotected with an equivalent molar concentration of NaOH for an hour before being mixed with DHLA and the solution was stirred for 4 hours. DHLA-NTA was prepared as described.⁵³ The final NTA-modified AuNPs were washed with water three times and purified using centrifugation with a centrifugal membrane filter (Millipore, 50–100 K molecular weight cut-off membrane filter) to remove free unbound ligands. High resolution transmission electron microscopy (TEM) images were taken to confirm both the size and standard deviation of the AuNPs with a sample size of approximately *n* = 100 for each distinct AuNP size (5, 10, and 20 nm).

AuNPs were loaded with Ni²⁺ for metal affinity coordination driven binding to the PTE₃’s terminal His₆ motifs using a cation exchange column procedure. Briefly, the cation exchange column (10 cm length × 1 cm diameter) was pre-
Dynamic light scattering (DLS) measurements were carried out using a ZetaSizer NanoSeries equipped with a HeNe laser source (λ = 633 nm) (Malvern Instruments Ltd, Worcestershire, UK) and an avalanche photodiode for detection, controlled with DTS software. 10 nM concentration solutions of AuNPs, PTE3-conjugated AuNPs or equivalent amount of PTE3 were loaded into disposable cells and data were collected at 25 °C. Three runs of the measurements were performed for each sample to achieve the zeta potential. All the samples were prepared in 0.1× PBS buffer pH 7.4.

**Transmission electron microscopy**

Structural characterization of as-prepared NPs was carried out using a JEOL 2200-FX analytical high-resolution transmission electron microscope (TEM) with a 200 kV accelerating voltage. Samples for TEM were prepared by spreading a drop (5–10 µl) of the filtered NPs dispersion (filtered using 0.25 µm Millipore syringe filters) onto ultrathin carbon/holey support film on a 300 mesh Au grid (Ted Pella, Inc.) and letting it dry. The concentration of NPs in the deionized water used was typically ~1–10 nM. Individual particle sizes were measured using a Gatan Digital Micrograph (Pleasanton, CA); average AuNP diameters and their corresponding standard deviations were extracted from measuring the size of ~100 NPs from each sample size.

**Dynamic light scattering**

Dynamic light scattering (DLS) measurements were carried out using CM Ion Exchange Chromatography Resin (Biorad) and saturated with a 0.1 M NiCl₂ stock solution in water. The as-prepared NTA-modified AuNPs were added to the top of CM column and kept in the column for at least 30 min to promote the interaction between the Ni²⁺ and NTA on the surface of the AuNP. The Ni²⁺-NTA-modified AuNPs were eluted from the column using deionized water. The eluent solution was concentrated using a centrifugal membrane filter. For preparing PTE3–AuNP, 25 µL PTE3 of various molarities was allowed to self-assemble overnight with 25 µL of 5, 10, and 20 nm Ni²⁺-NTA-modified AuNPs to complete the binding of the PTE3 enzyme's His₆-tag to the Ni²⁺-NTA as schematically demonstrated in Fig. 1. The enzyme to NP ratios were created by mixing the following molar concentrations for the 5, 10, and 20 nm sized AuNPs. For the 5 nm AuNPs, 3 picomoles of AuNPs were mixed with 0 to 54 picomoles of PTE3 to achieve PTE3–AuNP ratios of 0 to 18 respectively while the final concentration of AuNPs in each sample was 120 nM. For the 10 nm AuNPs, 0.5 picomoles of AuNP were mixed with 0 to 5 picomoles of PTE3 to achieve PTE3–AuNP ratios of 0 to 10 PTE while the concentration of AuNP in each sample was 25 nM. For the 20 nm AuNPs, 0.25 picomoles of AuNPs were mixed with 0 to 2.5 picomoles of PTE3 to achieve PTE3–AuNP ratios of 0 to 10 while the final concentration of AuNPs in each sample was 5 nM.

**Zeta-potential**

For zeta-potential (ζ-potential) measurement, Laser Doppler Velocimetry (LDV) measurements were performed using a ZetaSizer NanoSeries equipped with a HeNe laser source (λ = 633 nm) (Malvern Instruments Ltd, Worcestershire, UK) and an avalanche photodiode for detection, controlled with DTS software. 10 nM concentration solutions of AuNPs, PTE3-conjugated AuNPs or equivalent amount of PTE3 were loaded into disposable cells and data were collected at 25 °C. Three runs of the measurements were performed for each sample to achieve the zeta potential. All the samples were prepared in 0.1× PBS buffer pH 7.4.

**Enzyme assays**

The insecticide paraoxon was used as a model OP substrate for the PTE3 assays. PTE3 hydrolyzes the phenol groups yielding a p-nitrophenol product which has a characteristic absorption centered at 405 nm (molecular extinction coefficient of ~18 000 M⁻¹ cm⁻¹). Experimentally, varying molar ratios of PTE3 were functionalized to the 5, 10, and 20 nm AuNPs. Two experimental formats were utilized. Fixed amounts of AuNPs where the molar ratio of PTE3 per AuNP was systematically increased and fixed concentrations of PTE3 where the amount of AuNPs was systematically decreased to increase the ratio of PTE3/AuNP. Bioconjugate activity was assayed and compared to free PTE3 using a similar method as previously described.44 It should be noted that the AuNP–PTE3 ratios chosen were far below surface saturation in most cases to ensure that all enzymes were attached to the AuNPs. Conjugates and enzyme only controls were diluted into a final concentration series of paraoxon ranging from 20 to 5000 µM within 384-well Corning flat bottom, non-binding microtiter plates.45 Assays were carried out in a Tecan Infinite M1000 dual monochromator multifunctional plate reader using a xenon flash lamp (Tecan, Research Triangle Park, NC) to measure the absorbance at 405 nm every 20 seconds. The absorbance values were converted to p-nitrophenol concentrations via a standard calibration curve of p-nitrophenol ranging from 0 to 200 µM.46 While there is a small overlap in extinction spectrum of colloidal AuNP and p-nitrophenol, the contributions of AuNP accounts for less than 0.1% of the total optical density due to the small concentration used (i.e., optical density originating from the 5, 10, and 20 nm AuNPs amounted to 0.002, 0.002, and 0.002%, respectively).

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0.013%, and 0.07% respectively as compared to the total optical density of each solution via UV-Vis spectrophotometer measurements). Therefore, the extinction spectrum of colloidal AuNPs is negligible as compared to the extinction spectrum of p-nitrophenol. All assays were performed twice and average values utilized with standard deviations where appropriate. Initial rates of PTE3 activity and kinetic parameters were determined using the enzyme kinetics module within the SigmaPlot software which uses a non-linear curve fit for a single substrate.

Results and discussion

PTE3–AuNP synthesis and assembly

We have previously reported the enhancement of PTE and the PTE3 trimer when attached to Cd-containing semiconductor QDs14,20 and in this work the PTE3 trimer is attached to a nontoxic, Cd-free AuNP. The PTE3 trimer was prepared by appending the PTE gene to a collagen-derived triple helix domain and a trimerizing V domain followed by a terminal His6 motif as described in the Methods. The PTE-collagen-His6 monomer has an estimated $M_w$ of $\sim 53$ kDa while the fully assembled PTE3 trimer has an estimated $M_w$ of $\sim 155$ kDa. The PTE3 trimer was immobilized onto AuNPs of distinct diameter (i.e., 5 nm, 10 nm, and 20 nm) which were synthesized as described in the Methods. The native citrate stabilizing ligand was capped exchanged with a mixture of 50:50 DHLA and DHLA-NTA and that the NTA binding moiety is equally displayed around the AuNPs, with only the far smaller His6 motifs bind. This also assumes no steric hindrance between neighboring proteins surface where only the far smaller His6 motifs bind. This also assumes no steric hindrance between neighboring proteins and that the NTA binding moiety is equally displayed around the AuNPs in sufficient numbers to facilitate such a dense binding.49 This is, for all intents and purposes, the same interaction and mechanism by which the proteins were originally purified using commercial IMAC media. This approach allows for ratiometric control over the number of PTE3 assembled to each AuNP simply by varying the molar amounts of each mixed together. It also allows for orientational control, i.e. displaying the active site away from the NP surface, a factor that is important for minimizing the conjugate's structural heterogeneity which can, in turn, detrimentally decrease enzyme activity.48 The PTE-His6 motif, attached to the C-terminal of each of the three monomers, allows for the enzymatic binding pocket to orientate itself on the outward side of the enzyme–NP bioconjugate. Although each PTE3 construct will ultimately display $3 \times$ His6 on their termini due to its trimeric nature, functional results show no evidence of crosslinking between the AuNPs, but rather excellent conjugate stability presumably due to the cumulative-synergistic nature of the multiple interactions at the NP interface.

AuNP and PTE3–AuNP bioconjugate characterization

To confirm the size of the as-synthesized AuNPs, high resolution TEM images were collected from each sample batch (Fig. 2, left). As confirmed in the representative TEM micrographs shown in Fig. 2, the sample sizes ($n \sim 100$) corresponded to 5 ± 0.9 nm, 10 ± 1.4 nm and 20 ± 3.0 nm diameter AuNPs. This, in turn, corresponds to approximate surface areas of $\sim 79$ nm$^2$, $\sim 314$ nm$^2$, and $\sim 1257$ nm$^2$ along with estimated curvatures of $\sim 0.4$ nm$^{-1}$, $\sim 0.2$ nm$^{-1}$, and $\sim 0.05$ nm$^{-1}$, respectively. Structural modeling, similar to that described previously,14,23 was used to estimate maximum enzyme packing densities of ca. 30, 50, and 120 PTE3 that should fit onto the 5, 10, and 20 nm AuNPs. These large packing numbers result from the elongated PTE3 structure which places the large globular enzymes quite distal from the tight constraints of the NP surface where only the far smaller His6 motifs bind. This also assumes no steric hindrance between neighboring proteins and that the NTA binding moiety is equally displayed around the AuNPs in sufficient numbers to facilitate such a dense binding.49

Agarose gel electrophoresis was next conducted to confirm PTE3 physically assembled to the AuNPs with ratiometric display (Fig. 2, right). Incrementally increasing ratios of PTE3/AuNP were assembled to the NPs overnight as described in the Methods and loaded into 1% agarose gels for electrophoretic separation. The gels were visualized at 5 minutes intervals on a lightbox to monitor the effects of PTE3–AuNP conjugation on

Fig. 2 Left: Representative high resolution transmission electron microscopy (TEM) micrographs of the 5, 10, and 20 nm AuNPs. Right: Agarose gel electrophoresis analysis of three different-sized AuNPs assembled with varying ratios of PTE3 as indicated at the top. Images were collected every 5 min during separation.
migrate fastest towards the anode due to the high net negative charge from the DHLA and DHLA-NTA carboxyl endgroups. As the ratio of assembled PTE₃–AuNP increases, the resulting migration of the complexes decreases in a manner that is directly proportional to the enzyme–AuNP ratio. This is a direct result of the increase in mass and hydrodynamic size along with changes to the overall net charge of the complex. The latter are quite complex to predict due to the different sizes and surface areas involved. The smaller 5 nm AuNPs displayed the largest change in migration as expected since their size is closest to that of the enzyme. Overall, this confirms that the PTE₃ does assemble to these NPs with sufficient affinity to maintain the structure while being driven through a sieving matrix. Moreover, the assembly occurs in a direct ratiometric manner.

DLS and zeta potential were also utilized to monitor the increase in hydrodynamic diameter (H_D) of the AuNP as a function of the increase in attached PTE₃. The hydrodynamic size and the zeta potential of the AuNPs were changed after conjugation of PTE₃, which confirmed that the conjugation was achieved after 2 hours of incubation. A H_D of 10.1 ± 0.65 nm, 17.1 ± 1.7 nm, and 50.5 ± 1.93 nm was measured for the initial 10 nm AuNP before functionalization, after DHLA/DHLA-NTA functionalization, and after conjugation with PTE₃ in a molar concentration ratio of 32:1 with the AuNPs, respectively. The measured hydrodynamic size of the PTE₃-conjugated AuNP was slightly larger than expected, which perhaps is due to the mild aggregation in the solution caused by excess amount of unbound PTE₃. The zeta-potential value of the 10 nm AuNP for the same scenario decreased from −9.8 ± 1.72 for the AuNP (10 nm) before functionalization and to −28.7 ± 1.14, and −12.0 ± 1.5 mV after functionalization with DHLA/DHLA-NTA and PTE₃ (32:1 PTE₃ to AuNPs) respectively which suggests that the negatively charged surface of the AuNP becomes more charged with the DHLA/DHLA-NTA functionalization but less so with enzyme conjugation. The inclusion of the enzyme increased the hydrodynamic drag (∼17 nm to ∼50 nm) which may have reduced the mobility of the particle in the electrophoretic field neutralizing, to some degree, the zeta potential.

**PTE₃–AuNP activity characterization via fixed AuNP concentration method**

Full enzyme progress curves were first acquired by fixing the concentration of AuNPs while increasing the bioconjugate molar ratio of PTE₃ displayed around the AuNP. This “fixed AuNP concentration method” was carried out with 5, 10, and 20 nm AuNP scaffolds at 0.5 pm (which corresponds to a gold mass concentration of 0.0008%, 0.006%, and 0.05%, respectively) while the PTE₃ concentration was varied to yield distinct AuNP–PTE₃ ratios (Fig. 3). The kinetic activity of the said PTE₃–AuNP bioconjugates was tested and compared to an equivalent amount of control free PTE₃. The resulting changes in absorbance from these experiments (see ESI Fig. 1S and 2S†) were converted into product concentration by linear interpolation to a p-nitrophenol calibration curve (Experimental methods). The initial rate of paraoxon hydrolysis for each of the PTE₃–AuNP conjugates and corresponding controls of free enzyme were then plotted against the substrate concentration and fitted with the Michaelis–Menten equation, see Fig. 3a. The corresponding $V_{\text{max}}$, $k_{\text{cat}}$, and $K_M$ values for each of the assemblies and controls were also estimated from these data (Fig. 3b–d). Given the large concentration difference between AuNP conjugates and substrate concentration, we note that in principle the experimental format meets standard Michaelis–Menten and Briggs–Haldane assumptions.51

The ratios of PTE₃–AuNP utilized here were chosen so as to approach maximum packing for the smaller AuNP and then subsaturation for the larger materials. As expected, increasing PTE₃ concentration, both assembled on and off the AuNPs, increased the apparent $V_{\text{max}}$ values, as more enzyme was able to hydrolyze more substrate (Fig. 3b). For all the AuNP sizes and ratios tested, PTE₃ immobilization increased the observed $V_{\text{max}}$ At lower ratios of PTE₃–AuNP, the relative improvement in $V_{\text{max}}$ versus free enzyme was substantially higher displaying a nearly 17-fold increase at a 1:1 assembly ratio on the 20 nm AuNPs. Increases of 6.5-fold and 10.5-fold were noted for the 10 and 5 nm particles at the same ratio. On the other hand, when the ratio of PTE₃–AuNP increased to 24, $V_{\text{max}}$ only increased by approximately one third over the rate of free enzyme.

Comparing between the different size AuNPs, PTE₃ immobilization on the 20 nm AuNPs demonstrated an average enhancement (bioconjugate/free) of ∼1.7× and ∼1.1× higher activity or turnover rate than the same PTE₃–AuNP ratio of 10 and 5 nm, respectively. The catalytic rate ($k_{\text{cat}}$) of PTE₃ not attached to AuNPs was averaged in order to get a truer catalytic rate of free enzyme. As $k_{\text{cat}}$ ($V_{\text{max}}/\text{[PTE₃]}$) is directly correlated to $V_{\text{max}}$, there is an identical increase in $k_{\text{cat}}$ for PTE₃ attached to 5, 10, and 20 nm AuNP. As illustrated in Fig. 3c, $k_{\text{cat}}$ for PTE₃–AuNP is roughly constant, irrelevant of the concentration of the enzyme for each sized AuNP with the average value of 11.0, 16.0, and 17.3 s⁻¹ for 5, 10, and 20 nm, respectively, which is a ∼1.7× (20/5 nm) and ∼1.1× (20/10 nm) improvement. While $V_{\text{max}}$ and $k_{\text{cat}}$ show a significant improvement for PTE₃ immobilized on the AuNP, the Michaelis constant $K_M$ an indirect measure of enzyme–substrate affinity, remains similar to or higher than the free enzyme, suggesting that PTE₃ immobilization on the AuNP tends to decrease enzyme–substrate affinity. However, it should be noted here that the measured $K_M$ values for enzyme–NP conjugates are approximations at best since the enzyme–substrate complex cannot be strictly modeled as freely diffusing in solution. Moreover the Michaelis model overestimates the number of diffusing multivalent NP conjugates as well as underestimates the local concentration of enzyme in the presence of the NP.19,20,25,34 We do note that similar increases in $K_M$ values have been noted for attaching other enzymes to the NPs.30 Finally, the catalytic efficiency of immobilized PTE₃ ($k_{\text{cat}}/K_M$) is decreased when on the 5 nm AuNPs, ∼0.70×, however, increased by ∼3.0× and ∼1.2× when immobilized on the 10 and 20 nm AuNPs respectively.
Interestingly, despite the smaller surface curvature of the larger AuNPs, which may impede enzyme conformational changes and increase the likelihood of detrimental enzyme neighbor interactions, enzyme activity appears to be most enhanced on the larger 20 nm AuNPs. This result is counter to some of our previous observations where smaller NPs manifested better enhancement of enzyme activity following attachment.14,20 However, this previous work primarily utilized direct enzyme attachment to the ZnS surface of CdSe/ZnS core/shell QDs while in this work both the NP constituents and bioconjugation chemistry are distinct. This suggests that attachment chemistry and NP material type plays a role in enhancing the enzymatic behavior for distinct enzyme–NP constructs.

**PTE3–AuNP activity characterization via fixed PTE3 concentration method**

Full enzyme progress curves (see ESI Fig. 3S, 4S, and 5S†) were next acquired by fixed concentration of PTE3 while increasing the bioconjugate molar ratio of PTE3–AuNP by decreasing the AuNP concentration. This “fixed PTE3 concentration method” was developed by fixing the PTE3 concentration at 1.0 pM, while the concentration of the 5, 10, and 20 nm AuNPs were varied. This assay was performed to validate and cross compare to the previous “fixed AuNP concentration method” assay and subsequently the same enzyme performance experiments were performed in this assay as was performed in the previous. The resultant initial rate of paraoxon hydrolysis by the PTE3–AuNP bioconjugates was plotted against paraoxon substrate concentrations (Fig. 4a). Again, when PTE3 is displayed on the AuNP, most of the bioconjugates have improved initial velocities and this is also especially true for the bioconjugates at lower ratios of PTE3/AuNP.

The corresponding \( V_{\text{max}} \), \( k_{\text{cat}} \), and \( K_M \) values for each of the assemblies and control were also estimated from the fixed PTE3 concentration method assay and are compared as a
function of ratio in Fig. 4b–d. Fig. 4b highlights the enhancements in $V_{\text{max}}$ that were observed. In almost every case the incorporation of AuNPs increases $V_{\text{max}}$ with higher enhanced activity at lower ratios (1 : 1) and a slight decrease in activity at high ratios (1 : 32), 2.3× and 0.75× respectively for 20 nm AuNP. The same trends noted for $V_{\text{max}}$ values on the AuNP are also observed in the derived $k_{\text{cat}}$ values, Fig. 4c. Although significantly higher in value than the free enzyme control, $k_{\text{cat}}$ appears to decrease as the ratio of PTE3–AuNP is increased. There is also an increase in relative $k_{\text{cat}}$ as the AuNP increases in size from 5, 10 and 20 nm. Similar to results found in the fixed AuNP concentration method, $K_{\text{M}}$ maintains a fairly consistent value for PTE3 immobilized onto the 5 and 10 nm AuNPs but increases (enzyme affinity worsens) for various PTE3 concentrations on the 20 nm AuNPs. Resultant enzyme efficiency ($k_{\text{cat}}/K_{\text{M}}$) calculations show an increase (5.0×) for the 5 nm AuNPs but a decrease on the 10 and 20 nm AuNP (0.68× and 0.67× respectively).

**Conclusions**

In this work, we demonstrated that PTE3 enzymatic activity can be significantly enhanced when immobilized onto AuNPs. Results demonstrated $V_{\text{max}}$ could be enhanced nearly 17-fold by immobilization on the 20 nm AuNP at low concentrations of PTE3 (5 pM) and low ratio of PTE3–AuNP (1 : 1). These results corroborate and exceed our previous results where PTE3 performance was enhanced when attached to multiple sized QDs—upwards of four-fold improvement in $k_{\text{cat}}$ for PTE3 bound to 525 nm QDs versus 0.25 µM min$^{-1}$ mg$^{-1}$ for free glucose oxidase. Other researchers have explored the enhanced activity of enzymes immobilized to AuNPs and have observed similar results. For example, glucose oxidase immobilized on a 5 nm AuNPs displayed increases an order of magnitude higher $V_{\text{max}} = 1.42$ µM min$^{-1}$ mg$^{-1}$ immobilized on AuNP versus 0.25 µM min$^{-1}$ mg$^{-1}$ for free glucose oxidase. Enhancement of other enzymes such as glycosylated beta-galactosidase, which is commonly

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Fig. 4 Representative PTE3 enzymatic activity with fixed enzyme concentrations. Experiment conducted with constant PTE3 concentration while varying the AuNP to PTE3 ratio for the 5 nm, 10 nm, 20 nm AuNPs. (a) Initial rates with constant PTE3 concentration as AuNP concentration decreased. Lines are fits to the data using the Michaelis–Menten formula while the symbols represent experimental data. Solid lines and squares are PTE3 attached to the AuNPs while the dotted line and black triangles correspond to the identical concentration of PTE3 alone. Comparison of the (b) $V_{\text{max}}$, (c) $k_{\text{cat}}$ and (d) $K_{\text{M}}$ values derived from the data in (a) across all the various ratios of enzymes used. Blue bars represent PTE3 attached to AuNPs and red bars represent equivalent PTE3 free in solution. The error bars are the standard error from estimating the indicated values using repeated data.
used in lactose sensing, yielded over 10-fold increase in $V_{\text{max}}$ (soluble enzyme 0.58 µmol min$^{-1}$ mL$^{-1}$ and immobilized on AuNP 6.18 µmol min$^{-1}$ mL$^{-1}$).52

The number of PTE$_3$ attached to the AuNP affects the observed activity of the enzyme as loading more PTE$_3$ onto the AuNP decreases the relative magnitude of the enhancement. For example, PTE$_3$ displayed a significantly higher catalytic rate when immobilized at a 1:1 ratio of PTE$_3$/AuNP, but then showed a distinct decrease until the final 32:1 ratio (Fig. 4). PTE$_3$ enzyme immobilized on the larger 20 nm also displayed the most significant increase in catalytic activity ($k_{\text{cat}}$) values with a $\sim 1.7 \times$ and $\sim 1.1 \times$ increase as compared to the same molar ratio of enzyme immobilized on the 5 and 10 nm diameter AuNP, respectively. The origins of these patterns are not easily dissected out of the current data. As mentioned, the observation of the largest enhancement in enzyme performance is noted where the lowest molar ratios of PTE$_3$ are immobilized on the NPs—a behavior which mimics our previous reports on improving PTE$_3$ performance via immobilization on QDs.14 Researchers postulate that the immobilization of enzymes in lower density on NPs limits enzyme-to-enzyme neighbor interactions and hence increases the likelihood of proper enzyme conformation and subsequently higher enzyme activity or more specifically increases in the rate of enzyme–substrate to product conversion ($k_{\text{cat}}$).20,24,44 However, our results also demonstrate that PTE$_3$ activity is higher on larger sized NPs (i.e., 20 nm vs. 10 nm and 5 nm) which we previously postulated may arise in part to the high localized density of PTE$_3$ on the larger NPs and overall increases in avidity of the enzyme–NP bioconjugate.20

We do note a subtle but rather interesting discrepancy between the two experimental formats utilized here. In the first fixed AuNP concentration format, enzyme efficiency ($k_{\text{cat}}/K_M$) decreased on the 5 nm AuNPs and increased when the enzyme was immobilized on the larger NP materials. In the second fixed enzyme format, the converse of this pattern is observed. The origin of this pattern is not yet understood; however, we hypothesize that it is associated with enzyme packing and fitting on the NPs which could be far more constrained for smaller materials in the former experimental format. Moreover, investigation of beta-galactosidase activity when assembled on QDs also suggests the possibility of substrate accumulation or sequestration in the NP interface,53 and this, too, would be constrained or limiting for smaller materials in the first experimental format by the increasing amount of enzyme present. Lastly, it is again worth pointing out the complexities of this interfacial environment and how much still remains unknown about it. Clearly, it is not unreasonable that subtle changes in experimental formats such as variations in surface chemistry; enzyme position/orientation and concentration; and substrate-to-NP attraction/diffusion rates could give rise to changes in the enzyme–NP structure and efficiency in which, in turn, changes the observed activity.54

Clearly, far more work will be needed to elucidate the underlying mechanism(s) and nuances that give rise to the enhancement of enzymatic activity at a NP interface. These include surveying a far larger size range of NP materials, a range of NP surface chemistries, different types of enzymes and assays, and different types of NP bioconjugation chemistries. What these types of studies will ultimately provide, is insight into the nature of the NP–enzyme interface and how this environment gives rise to enzymatic enhancement. Critically, the nanoscale interfacial environment, including the substrate and water boundary layers and gradients, immediately surrounding the enzyme–NP bioconjugate are postulated phenomena that gives rise to such enzymatic enhancement.23 Unfortunately, there are almost no metrologies currently available to probe and characterize this nanoscale interfacial environment,55 leaving the systematic and parametric functional assays suggested above as the more realistic but still indirect way forward.

Finally, the lower cost, facile fabrication protocol, and environmentally benign nature of AuNPs make them well suited as a scaffold that hosts and augments enzymes for rapid in-field OP sensors along with giving them excellent potential for incorporation into protective materials. A simple in-field OP sensor based on the PTE$_3$–AuNP could be fabricated into a fluorescence sensor utilizing a smartphone in a single step readout.56 Similar photoluminescence sensors have been shown for point-of-care measurements of hydrolase activity in serum and whole blood using semiconductor QD FRET-based detection.7 Furthermore, this work adds to the ever growing body of NP–enzyme bioconjugates and suggests the application of NP–enzyme conjugates for medical, research, industrial and commercial utility which would certainly benefit from enhanced and accelerated enzymatic activity.25,57,58 Such enzyme–AuNP bioconjugates, and especially the phenomena that they exploit, could have a significantly broad impact on a variety of fields beyond biosensing to include environmental remediation, synthetic biology and even batch chemical processing.59

Acknowledgements
J. C. C and I. L. M gratefully acknowledges that this material is based upon work that is supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, under award number 11901762. J. C. C. also acknowledges support from the Iowa State University College of Engineering and Department of Mechanical Engineering while I. L. M. also gratefully acknowledges support from ONR, NRL, and the NRL-NSI.

Notes and references