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A Robust and General Approach to Quantitatively Conjugate Enzymes to Plasmonic Nanoparticles

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ABSTRACT: Bioconjugates of plasmonic nanoparticles have received considerable attention due to their potential biomedical applications. Successful bioconjugation requires control over the number and activity of the conjugated proteins and the colloidal stability of the particles. In practice, this requires reoptimization of the conjugation protocol for each combination of protein and nanoparticle. Here, we report a robust and general protocol that allows for the conjugation of a range of proteins to different types of nanoparticles using very short polyethylene-glycol (PEG) linkers, while simultaneously preserving protein activity and colloidal stability. The use of short linkers ensures that the protein is located close to the particle surface, where the refractive index sensitivity and near-field enhancement are maximal. We demonstrate that the use of a Tween20 containing stabilizing buffer is critical in maintaining colloidal stability and protein function throughout the protocol. We obtain quantitative control over the average number of enzymes per particle by either varying the number of functional groups on the particle or the enzyme concentration during incubation. This new route of preparing quantitative protein-nanoparticle bioconjugates paves the way to develop rational and quantitative strategies to functionalize nanoparticles for applications in sensing, medical diagnostics, and drug delivery.

INTRODUCTION

Plasmonic noble metal nanoparticles are suitable for a large variety of scientific and commercial applications, such as sensing, spectroscopy, and energy conversion, due to their outstanding optical properties. These optical properties are governed by the unique localized surface plasmon resonance (LSPR), which are essentially coherently oscillating electrons in the conduction band induced by radiation with an electromagnetic wave. The optical properties of plasmonic particles are easily fine-tuned by changing their size, shape, and material via various well-developed wet chemistry protocols, leading to a rich library of available plasmonic nanoparticles directly for research. One of the most important consequences of LSPRs is the localized electromagnetic field around the nanoparticles, which causes plasmon resonance peak shifts in response to local refractive index changes. Plasmonic particles can also act as antennas to modify the photophysical properties of single fluorescent molecules by modulation of the excitation rate, radiative, and nonradiative decay rate. Plasmonic particles are therefore ideal candidates for enhanced molecular detection and are indispensable tools in single-molecule imaging and biosensing.

Bioconjugates of plasmonic particles have therefore received considerable attention, for example, particles functionalized with proteins, peptides, or oligonucleotides are increasingly found as important players in biosensors, nanocarriers, and nanotherapeutics. Nevertheless, complex molecular interactions exist at the interface between the nanoparticle and the conjugated biomolecules. The colloidal stability of the nanoparticles, the hydrodynamic behavior of the suspending medium, and the folded structure of the biomolecules all play pivotal roles in successful bioconjugation. Several challenges are associated with the controlled preparation of nanoparticle bioconjugates, especially via a robust and general protocol. To begin with, there is a large diversity of surface ligands including surfactants and polymers that keep the as-synthesized nanoparticles colloidally stable. In practice, this requires a tailored ligand exchange protocol to keep each type of particle stable during the conjugation. Second, quantitative determination of the number of biomolecules per nanoparticle is inherently difficult because reaction stoichiometry often does not directly relate to the added molar ratio of nanoparticle and biomolecule. Third, the inherent dispersion in nanoparticle size and ligand density could complicate the control over colloidal stability and biomolecular display on nanoparticles.

Various strategies to conjugate biomolecules to colloidal nanoparticles have been proposed. Biomolecules can be directly adsorbed onto the nanoparticle’s surface by physisorption. However, physisorption often leads to (local)
unfolding of the biomolecules resulting in significant loss of bioactivity. Bioconjugation via a ligand or a polymeric linker is widely used to avoid direct contact of the biomolecules with the nanoparticle surface. Standard bioconjugation techniques have been introduced using bifunctional cross-linkers. Electrostatic adsorption via linkers with charged end groups has been shown to effectively conjugate proteins to gold nanoparticles. Multibranched polymers and dendrimers have also been used for bioconjugation via various surface chemistries and have been particularly useful to functionalize particles for drug delivery and applications in undiluted complex media. The most widely used covalent approach uses N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) and its derived cross-linkers connecting primary amines on the biomolecule with carboxyls on the particle via amide bonds. Both gold and silver nanoparticles have been conjugated to proteins and peptides using such EDC-mediated protocols. However, the required strict control of pH and ionic strength leaves only a narrow window in which the particles remain colloidal stable, and this window varies from colloid to colloid depending on the charge and type of capping ligand. Bio-orthogonal approaches using click chemistry have shown to allow accurate quantitation of the average number of biomolecules per particle. However, click reactions rely on hydrophobic azide or alkyne groups on either the particle or on the biomolecule, which often leads to reduced solubility and colloidal stability in aqueous environments. Conjugation via biotin-streptavidin linkers has been used in the study of nanoparticle-based biosensors on ensemble and single-molecule levels and the study of nanoparticle assemblies. Being a water-soluble small molecule, biotin is typically conjugated to the surface of nanoparticles via poly(ethylene)-glycol (PEG) cross-linkers, without significant aggregation of the nanoparticles. However, maintaining colloidal stability requires long PEG linkers to prevent aggregation during centrifugation or at physiological salt conditions, pushing the conjugated biomolecules away from the particle’s surface where the refractive index sensitivity and fluorescence enhancements are highest. Although it has been observed that histidine-tagged biomolecules can effectively conjugate to inorganic quantum dots with zero-length spacing, no general protocols exist that allow for the conjugation of a range of proteins to different types of plasmonic nanoparticles using short linkers while simultaneously preserving protein activity and colloidal stability.

In this manuscript, we demonstrate a general and robust approach to quantitatively conjugate a range of enzymes to a range of metal nanoparticles using short linkers. Owing to the different stabilizing ligands employed during the synthesis of the particles, we demonstrate that the use of a Tween20 containing stabilizing buffer is critical in maintaining colloidal stability and protein function throughout the functionalization process. To show the generality of the approach, we conjugate alkaline phosphatase (ALP), β-galactosidase (β-gal), and horseradish peroxidase (HRP) to as-synthesized gold nanospheres, gold nanorods, and silver nanospheres that each display different surface ligands and different ζ-potentials. We obtain quantitative control over the average number of enzymes per particle by changing either the number of functional groups on the particle or by changing the enzyme concentration during incubation. Quantitative conjugation of proteins to nanoparticles will enable the use of these bioconjugates in nanomedicine, cellular targeting, and biosensing. Although displayed with diluted buffers, our method is not limited by real undiluted samples, such as serum and whole blood, due to the high conjugation specificity and detection sensitivity.

### EXPERIMENTAL SECTION

#### Nanoparticle PEGylation.

Gold and silver nanospheres (40 nm in diameter) stabilized in sodium citrate buffer were purchased from Sigma-Aldrich. Gold nanorods (60 × 30 nm², extinction peak 610 nm) were purchased from Nanoseedz. The gold and silver nanospheres were directly used for PEGylation, whereas gold nanorods were centrifuged once and resuspended in an aqueous solution containing 1 mM CTAB. SH-PEG-biotin (1 kDa) was purchased from Nanocs and SH-PEG-methyl (800 Da) was purchased from Sigma-Aldrich. Stock solutions of PEG were aliquoted at 2 mM in MQ water and stored frozen at −20 °C. The mixed solutions of PEG with varying fractions (φ) of SH-PEG-biotin were then prepared in 10 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) solution in MQ water to prevent formation of disulfide bridges.

For PEGylation, 100 μL of nanoparticle solution was centrifuged in to a pellet (9000 rpm, 10 min), and a 100 μL solution of the mixed PEG-solution at 1 mM total concentration was added to the pellet, vortexed, and incubated for at least 4 h. After PEGylation, the PEGylated nanoparticles were centrifuged and redispersed at least 5× in a Tris or PBS buffer with 0.1% of Tween20 added to it. The addition of Tween20 was crucial as it ensured colloidal stability even before centrifugation or at physiological salt conditions, pushing the conjugated biomolecules away from the particle’s surface where the refractive index sensitivity and fluorescence enhancements are highest. Although it has been observed that histidine-tagged biomolecules can effectively conjugate to inorganic quantum dots with zero-length spacing, no general protocols exist that allow for the conjugation of a range of proteins to different types of plasmonic nanoparticles using short linkers while simultaneously preserving protein activity and colloidal stability.

#### Enzyme Conjugation.

Streptavidin-conjugated enzyme (β-galactosidase, alkaline phosphatase, and horseradish peroxidase) were obtained from Sigma-Aldrich. Stock solutions of enzyme were prepared in buffer. The buffer used for ALP was 25 mM Tris with 0.1 wt % Tween20 at pH 9, and the buffer for β-gal and HRP was 10 mM phosphate buffered saline with 0.1 wt % Tween20 at pH 7.4. For enzyme conjugation, stock solutions of the enzyme were added to PEGylated nanoparticle solutions to a final concentration of enzyme ranging from 1–100 mM. The nanoparticle-enzyme mixture was vortexed for 10 s and kept at −4 °C for at least 12 h before being centrifuged and redispersed at least 5× in the same Tween20 containing buffer. This ensured that nearly no free enzyme was left in the solution (see Figure S1 in the Supporting Information). Despite different ionic strength and pH of the buffers, we found that the use of Tween20 was again crucial to ensure colloidal stability.

#### Quantification of Conjugate Stoichiometry.

The prepared biocomplex was then ready for the determination of the final nanoparticle and enzyme concentration, the ratio of which provides the stoichiometric number of enzymes per particle. The former was determined by UV–vis extinction spectroscopy for which the extinction spectra of the nanoparticle suspensions were measured in a Shimadzu 2600 spectrophotometer in a quartz cuvette with an optical path length of l = 3 mm. The nanoparticle concentration was then determined by the Lambert–Beer law \[ A = \epsilon_c \times c \times l \] , where \( A \) is the measured extinction value and \( \epsilon_c \) is the molar extinction coefficient.

To determine the concentration of enzymes, the fluorogenic enzyme substrate 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO) was used for ALP and β-gal. DDAO phosphate and DDAO galactose were used as substrates for ALP and β-gal, respectively, and Amplex Red was used for HRP in the presence of hydrogen peroxidase. All enzyme substrates were purchased from Thermofisher. For DDAO-based enzyme reactions, the enzymes cleave the phosphate or galactose groups, leading to the recovery of DDAO fluorescence, whereas Amplex Red reacts with hydrogen peroxidase in the presence of HRP, producing highly fluorescent resorufin. Stock solutions of the substrates were prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C before dissolving in Tris or HEPES buffer.
PBS buffer for enzymatic assays. A Fluoroskan Ascent FL well plate reader (Thermo Fisher) was used to read out the fluorogenic enzymatic reactions from 96-well plates. In an enzymatic assay, a total volume of 100 μL of solution was pipetted into each well and mixed, after which the fluorescence signal was recorded as a function of time. An excitation/emission filter pair of 647/660 nm was used for DDAO-based reactions, and 560/580 nm was used for Amplex Red reactions.

## RESULTS AND DISCUSSION

In Figure 1a, we show the workflow of the bioconjugation in which the nanoparticles and nanorods with different native surface charges are used for conjugation with streptavidin-conjugated enzymes. Colloidal synthesis of plasmonic metal nanoparticles relies on the reduction of metal ions in aqueous solution, and the most widely used plasmonic nanoparticles prepared are gold and silver nanospheres and gold nanorods. For the synthesis of gold and silver nanospheres (AuNS, AgNS), citric acid is usually used as a reducing agent and as a stabilizer, preventing the aggregation of nanoparticles by a high density of negative charges on the surface.48,49 For the synthesis of gold nanorods (AuNR), high concentrations (>10 mM) of the surfactant CTAB have to be present acting as a shape regulator, guiding the growth of spherical seeds into elongated nanoparticles. As a result, the synthesized AuNRs are coated with a dense bilayer of positively charged CTAB molecules providing colloidal stability.

It is ideal to fully remove the citrate or CTAB in the nanoparticle solution, since biological applications of nanoparticles cannot be pursued with either citrate or CTAB at high concentration due to their cytotoxicity.50,51 A simple removal usually will cause aggregation because the surface charge that provides electrostatic stabilization is diminished. SH-PEGs and their derivatives are therefore often used to remove most of the surfactant and act as a steric stabilizer for the particles.5,45,52 PEGylation of nanoparticles has been widely reported in literature5,7,45,52 and from these studies, it is known that colloidal stability in high ionic strength solutions is only achieved with PEG linkers >5 kDa. To facilitate bioconjugation via shorter PEG linkers <1 kDa, we adapted the PEGylation protocol by Liao et al.45 and used a solution of mixed SH-PEG-

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**Figure 1.** (a) Workflow of the bioconjugation. Prior to the protocol, plasmonic nanoparticles (yellow) are stabilized by their native ligands (green) in an ionic buffer (gray). After centrifugation, the pellet from the native solution is incubated with a mixed PEG solution (cyan) for PEGylation. After PEGylation, the mixture is purified by centrifugation (5x) followed by redispersion in Tween20 containing buffer (blue), indicated as state II. Enzyme stock solutions (red) prepared in Tween20 containing buffer are then added to the mixture, followed again by incubation and purification by centrifugation (4x). The nanoparticle-enzyme bioconjugates are finally dispersed in Tween20 containing buffer, indicated by state III. (b) Sketches of the nanoparticle surface chemistry in states I, II, and III as shown in part a. In state I, the nanoparticles are stabilized by either the positively charged surfactant CTAB (AuNR) or by negatively charged citrate ions (AuNS and AgNS). In state II, the nanoparticles are stabilized by a mixed PEG layer (black) with exposed biotin groups (cyan) and Tween20 (dark blue). In state III, the enzyme is conjugated via biotin–streptavidin linkage. (c) Molecular structure of the thiolated PEG linkers and Tween20 used in the bioconjugation. The spherical shape of the nanoparticles is only for illustration and does not attempt to describe the true geometry.

**Figure 2.** UV–vis extinction spectra of (a) AuNR, (b) AuNS, and (c) AgNS before and after enzyme conjugation in states I (solid line) and III (dashed line). For comparison, we also show the spectra in state III where no Tween20 was used in the whole protocol. In the insets, we show the transmission electron microscope (TEM) images of the corresponding nanoparticles used in the conjugation.
biotin and SH-PEG-methyl in the presence of Tween20 to PEGylate and simultaneously biotinylate the surface of nanoparticles with a controlled fraction of biotin groups (Figure 1b). The use of a mixed PEG layer enables us to vary the number of biotin groups per particle while keeping the total PEG coverage constant. This allows us to directly compare the different functionalization protocols.

The use of short PEG linkers decreases the absolute distance between the molecule and nanoparticle surface, maximizing the refractive index sensitivity and fluorescence enhancements. In the ideal chain model, the end-to-end distance of a polymer chain scales as $\sqrt{N}$, with $N$ as the number of segments in the chain. Reducing the PEG length from 5 kDa to 1 kDa therefore reduces the time-averaged spacing between the biomolecule and the particle by ~2 nm, which is substantial considering the short length of the localized electric field around these small particles. As an example, the $1/e$ decay length of field intensity is about 5 nm for a gold nanorod with a diameter of 30 nm.46

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assuming Michaelis–Menten kinetics (see Figure S3 in the Supporting Information). As an example, we demonstrate the determination of the number ALPs per gold nanorod in Figure 3. The average number of ALPs per gold nanorod $N_{enz}$ is given by the fraction of the concentration of active enzymes $c_{enz}$ and the concentration of nanoparticles in the bioconjugate $c_{NP}$

$$N_{enz} = \frac{c_{enz}}{c_{NP}}$$

(1)

The effective concentration of conjugated enzyme, $c_{eff}$, was determined from the ratio between the initial velocity of the bioconjugate and the free enzyme at a known concentration, given by

$$c_{eff} = \frac{V_{0}^{conj}}{V_{0}^{free}}$$

(2)

where $V_{0}^{conj}$ and $V_{0}^{free}$ (in relative fluorescence units, RFU, per second) are the initial velocity of the bioconjugate and the free enzyme solution with a known concentration. In Figure 3a, we show the progress curves of 1 nM free streptavidin-conjugated ALP and the AuNR-ALP bioconjugate. The initial-phase of the progress curve is fitted with a linear equation in the form of $V = a + V_{0}t$, with the slope $V_{0}$ as the initial velocity. Significant nonlinearity of the progress curves sets in after 20 s due to substrate depletion. In this particular case, we find $c_{eff} = 10$ nM, which resulted in $N_{enz} = 12$ based on the nanoparticle concentration of 0.78 nM.

One question that remains to be addressed is whether the nanoparticle-conjugated enzymes are as active when conjugated to the nanoparticles as in their free state. More often than not enzymes conjugated to nanoparticles show significantly decreased activity due to conformational changes during, e.g., physisorption or covalent enzyme modifications that are unfavorable to maintain activity. In contrast, in some circumstances, an increased enzyme activity upon immobilization has been reported.\(^{1,6}\) The mechanism behind the nanoparticle-induced enhancement is complex as it involves the enzyme’s conformation, accessibility of the active site, and the local chemical environment (charge, pH) near the particle that may promote or inhibit activity. Nevertheless, it has been confirmed that the unique microenvironment surrounding the nanoparticles and the enzyme’s physicochemical interaction with the particle may modify the enzyme’s catalytic activity.

To assess the enzyme’s activity before and after conjugation, we performed a supernatant assay of the AuNR bioconjugates prepared with 50 nM ALP. In Figure 3b, we show the initial velocities of 5 nM free streptavidin-ALP and the sum of the activities of the bioconjugate after centrifugation and the supernatant. We observe that the enzyme activity of the bioconjugate was nearly 100% of the original activity. We attribute the high retention of activity to the dense PEG layer on the particle and on the noncovalent biotin–streptavidin interactions due to which very little to no modifications of protein structure are expected during the bioconjugation.

We now demonstrate that the number of enzymes per particle can be quantitatively controlled for different enzymes and particles by (1) controlling the enzyme concentration during incubation or (2) controlling the fraction of biotin groups in the mixed PEG layer. To demonstrate the effect of enzyme concentration used during the bioconjugation on $N_{enz}$ we measured $N_{enz}$ for bioconjugates prepared with a concentration series of ALP and $\beta$-gal. In Figure 4, we show the measured $N_{enz}$ determined for AuNR, AgNS, and AuNS coated with a mixed layer of PEG with a biotin fraction $\chi_b = 0.4$. As we can see in Figure 4a, $N_{enz}$ for nanoparticle-ALP bioconjugates can be controlled by the ALP concentration giving access to stoichiometries ranging from monovalent up to 20 enzymes per particle when the enzyme concentration was increased from 1 to 100 nM. Note that $N_{enz}$ is a measurement of active enzymes only and may not represent the true coverage of enzyme molecules since inactive enzymes do not contribute to the initial velocity, meaning that we are ‘blind’ in our measurement to the enzymes that are inactive already in the stock solutions. Nevertheless, the maximum value of $N_{enz}$ is limited by number of biotin groups and by the physical size of the enzyme compared to the particle. Despite their surface area, the number of enzymes on the AuNRs reached a plateau at the highest enzyme concentrations, whereas the coverage on the nanospheres was not saturated yet. We hypothesize that this might be due to the less efficient removal of CTAB from the AuNR surface leading to a lower number of biotin groups per particle.

The generality of the protocol is demonstrated in Figure 4b, where we show $N_{enz}$ for AuNR-$\beta$-gal and AuNR-HPR bioconjugates. We found that $N_{enz}$ for $\beta$-gal and HRP also scaled with enzyme concentration during incubation and reached approximately $N_{enz \text{ max}} = 10$ and 25, respectively, at 100 nM incubation. The lower $N_{enz \text{ max}}$ for AuNR-$\beta$-gal compared to ALP and HRP is likely due to the bigger physical size of $\beta$-gal (520 kDa) compared to those of ALP (140 kDa) and HRP (44 kDa), leading to a larger footprint of the protein.

Figure 4. (a) Number of ALP per particle as a function of ALP concentration for AuNR, AuNS, and AgNS bioconjugates. (b) Number of $\beta$-gal per particle as a function of $\beta$-gal concentration for AuNR bioconjugates. $\chi_b = 0.4$ is fixed during bioconjugation.
The theoretical maximum number of enzymes per particle $N_{\text{enc}}^{\text{max}}$ for ALP and β-gal can be estimated by assuming that the particles exhibit a perfectly spherical or spherically capped cylindrical geometry. These particle shapes exhibit a surface area $A_{\text{NP}}$ that for spherical particles can be written as $A_{\text{NP}}^{\text{sph}} = 4\pi r^2$ with $r$ as the particle radius. For a spherically capped cylinder, the surface area available for enzyme conjugation is given by $A_{\text{NP}}^{\text{cyl}} = 4\pi r^2 + 2\pi r(L - 2r)$, where $r$ is the radius of the rod and $L$ is its length. The maximum number of enzymes then follows from

$$N_{\text{enc}}^{\text{max}} = \frac{f A_{\text{NP}}}{A_{\text{enz}}}$$

(3)

where $A_{\text{NP}}$ is the surface area of the respective particle shape, $A_{\text{enz}}$ is the estimated footprint of a single enzyme molecule, and $f = 0.4$ is a correction factor to account for the fact that random sequential absorption leads to a maximum surface coverage of approximately 40%. The random sequential absorption describes a process where enzymes are introduced randomly, and irreversibly adsorb on a surface. It is commonly used to estimate the maximum coverage of spherical objects such as proteins on a surface. The footprint $A_{\text{enz}}$ of single enzymes without streptavidin was estimated using data from the protein databank, giving $A_{\text{enz}} = 4.52$ nm$^2$ for 520 kDa β-gal, $A_{\text{enz}} = 60$ nm$^2$ for 140 kDa ALP, and $A_{\text{enz}} = 31$ nm$^2$ for 44 kDa HRP. In Table 2, we show $N_{\text{enc}}^{\text{max}}$ based on these estimates. Our experimental $N_{\text{enc}}^{\text{max}}$ of up to 20, 11, and 24 for ALP, β-gal, and HRP, respectively, are thus reasonable numbers and indicate that the found maximum coverage is limited by the available surface area for random sequential absorption of the enzymes. Small deviations from the estimated maximum number could be due to the actual density of biotin on the nanoparticle surface, the presence of streptavidin, the size-dispersion of the particles, and electrostatic-repulsion between neighboring enzymes leading to a lower coverage than expected from the random sequential absorption model.

Finally, we demonstrate that the biotin fraction $\chi_b$ can also be used to tune $N_{\text{enc}}$. We prepared PEGylated AuNRs with $\chi_b$ ranging from 0.001 to 0.4 and used 10 nM ALP for bioconjugation. As shown in Figure 5, $N_{\text{enc}}$ increases from 1 to 9 with increasing $\chi_b$. This indicates that by increasing the number of biotins on the nanoparticle surface, the capture of the enzyme from the solution becomes more efficient leading to a larger number of enzymes per particle. The result also shows that SH-PEG-methyl (800 Da) provides excellent antifoiling because at low biotin fractions, we find a strongly reduced number of enzymes per particle, indicating little to no nonspecific binding of streptavidin-conjugated enzymes to SH-PEG-methyl.

As an outlook of our approach, we envision the resolution of the number and position of the conjugated enzymes by using microscopy methods. Transmission or scanning electron microscopes, for example, can be employed to perform this task after immunolabeling the enzymes with very small (<5 nm) metallic nanoparticles. The number and distribution of enzymes can then be visualized with the help of the high electron density of the labeling nanoparticles. Stochastic super-resolution microscopy based on sequentially blinking fluorophores can also be used as a powerful tool to localize single molecules on a single-particle basis and has been successfully performed on gold nanoparticles with high spatial resolution.

Understanding the number and distribution of enzymes on single particles is important in a number of applications. For example, it has been found that the density of enzymes on the nanoparticle surface directly impacts the enzyme activity, which needs to be taken into account when using enzymatic conversions for label-free analyte detection. Enzyme quantity and distribution were also established to be crucial in the performance of enzyme-powered nanomotors, where a certain threshold of enzyme numbers and asymmetric distribution was needed to generate nanoparticle motion.

**CONCLUSION**

We have developed a general approach to quantitatively conjugate enzymes to plasmonic nanoparticles including AuNRs, AuNS, and AgNS. The nanoparticles were first PEGylated with a mixture of SH-PEG-biotin and SH-PEG-methyl and then effectively stabilized in an enzyme reaction buffer containing Tween20. Then the PEGylated nanoparticles were conjugated to streptavidin-conjugated enzymes in the same buffer. We showed that our approach minimized particle aggregation and facilitated quantification of the number of enzymes per nanoparticle using fluorogenic enzyme assays, and we demonstrated that the bound enzymes maintained their biological activity. We could control the number of specifically bound enzymes per particle by varying the enzyme concentration and biotin fraction on the nanoparticle surface. Our protocol features high robustness and generality, due to the use of Tween20 as a stabilizing agent, and is highly versatile and flexible due to the use of commercially available mixed layers of PEG. This new route of quantitative protein-nanoparticle bioconjugate preparation that we demonstrated here may assist in the development of rational strategies to use nanoparticle bioconjugates in sensing, medical diagnostics, and other applications.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.9b01879.
Supernatant assay, extinction spectra of bioconjugates, free enzyme kinetics (PDF)

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**Notes**

The authors declare no competing financial interest.

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