Stabilization of Alkaline Phosphatase with Au@Ag₂O Nanoparticles

Brian A. Zaccheo and Richard M. Crooks*

Department of Chemistry and Biochemistry, Texas Materials Institute, and Center for Nano- and Molecular Science and Technology, The University of Texas at Austin, 1 University Station, A5300, Austin, Texas 78712-0165, United States

Supporting Information

ABSTRACT: Here, we report that a conductive Au@Ag₂O nanoparticle structure significantly enhances the stability of alkaline phosphatase (AlkP) in the presence of the inhibitors urea and l-phenylalanine (Phe). The enzyme/nanoparticle construct is prepared by associating the enzyme with citrate-capped Au particles, and then adding Ag⁺. UV–vis and XPS spectroscopy and transmission electron microscopy confirm the core@shell structure. AlkP activity was quantified in the presence and absence of the two inhibitors using a time-resolved colorimetric assay. The results indicate that 21% of the initial active AlkP is incorporated into the nanoparticle structure. More importantly, however, the Au@Ag₂O core@shell host reduces the inhibitory effect of urea and Phe by factors ranging from 3 to 12, depending on the inhibitor and its concentration, compared to the wild-type enzyme.

INTRODUCTION

Here, we report that alkaline phosphatase (AlkP) is stabilized against organic inhibitors by core@shell colloidal nanoparticles (NPs). These composites are prepared by combining AlkP and an Au colloid solution with Ag⁺. This results in agglomerated particles having an Ag₂O shell (AlkP-Au@Ag₂O), which stabilizes and protects the enzyme against inhibitors that would otherwise greatly reduce its activity. This finding is significant, because many applications are carried out under less-than-optimal conditions for enzyme functionality.

AlkP is a common enzyme that has previously been isolated and characterized. The wild type (WT) bovine intestinal isoform is an effective catalyst for the hydrolysis of phosphate groups present on a wide variety of substrates, has an approximate molecular weight of 60 kDa, and exhibits optimal activity under moderately basic conditions (pH 9–10). AlkP is often integrated into analytical assays and sensing schemes owing to its ease of handling and high catalytic activity. To improve the stability of AlkP and other enzymes used for applications like biosensing, there has been interest in encapsulating them within more rugged host materials. For example, Braun et al. encapsulated AlkP in a bulk sol–gel matrix that was subsequently pulverized and used as column packing material. AlkP in this matrix exhibited a 15% greater tolerance for incubation at 70 °C relative to free AlkP in solution. Smith et al. examined the activity of horseradish peroxidase (HRP) in sol–gel monoliths, coatings, and pulverized powders and reported that the powder form of HRP was active and reusable. These results are also important as they show sol–gel protein entrapment is not exclusive to AlkP. Sol–gels have also been used to protect proteins against drying. For example, Li et al. entrapped a fluorescent protein inside a sol–gel slab and reported that the protein remained fluorescent after lyophilization and rehydration. A loss of the fluorescence signal would indicate changes in the protein structure. The key point common to all of these examples is that sol–gel hosts have been shown to stabilize some proteins against common degradative practices including heating, aging, and drying.

The previously discussed sol–gel materials entrap enzymes within a network of pores. Alternatively, individual enzymes can be stabilized by other types of coatings that are directly attached to the enzyme. For example, Kim et al. modified surface amines on chymotrypsin with alkyl silanols to yield porous silicate gels around the enzyme. This treatment was shown to extend the active lifetime of the enzyme in solution. Thiogol groups were used in a similar way to reduce Au³⁺ onto α-amylose and form ∼30 nm NPs that retained amylase activity. Like sol–gels, the Au shell was determined to be porous by scanning for amylase activity using substrates with different molecular weights. HRP has been isolated within porous, hollow nanoshells. In this experiment, AgCl-entrapped HRP colloid was prepared inside reverse micelles. HAuCl₄ and a reducing agent were phase-partitioned sequentially into the micelles, and after purification of the particles, the AgCl was removed with ammonia to leave active HRP inside an Au shell. Ben-Knaz and Avnir reported that acid phosphatase could be stabilized inside a metal powder by incubating the enzyme in solutions of Ag⁺ or Au⁺ and adding Zn⁰ as a reducing agent.

In the present manuscript, we report the stabilization of AlkP against the inhibitory chemicals urea and l-phenylalanine (Phe) using composite NPs. We characterized these AlkP-Au@Ag₂O NPs using transmission electron microscopy (TEM), UV–vis spectroscopy, X-ray photoelectron spectroscopy (XPS), and energy dispersive spectroscopy (EDS). The results indicate that

Received: June 26, 2011
Revised: August 1, 2011
Published: August 16, 2011
AlkP strongly associates with Au colloids and that the addition of Ag⁺ to the enzyme-NP conjugate yields an Ag₂O shell. AlkP activity was quantified using a time-resolved colorimetric assay, and the data indicate that ~21% of the active enzyme initially present is associated with the AlkP-Au@Ag₂O composite. Finally, the activity of free AlkP was compared to that of AlkP-Au@Ag₂O in the presence of two inhibitors. Under otherwise identical conditions, AlkP-Au@Ag₂O was at least three times more active, which demonstrates the stabilizing effect of the Ag₂O shell. Two key advantages of the method described here, compared to previous literature reports, are that this approach uses a one-pot synthesis and the time required to stabilize the enzyme is less than one day.

**EXPERIMENTAL SECTION**

**Chemicals and Materials.** Citrate-capped Au colloids with a nominal diameter of 5 nm, Silver Enhancer Solution A (henceforth referred to as Ag⁺ solution); p-nitrophenol phosphate disodium salt hexahydrate, enzymology grade (pNPP); and L-phenylalanine (Phe), USP, were purchased from Sigma (St. Louis, MO). Calf intestinal AlkP solution was purchased from New England Biolabs, Inc. (Ipswich, MA). Urea, ACS grade, was purchased from Thermo Fisher Scientific (Waltham, MA). Diethanolamine, 99%, was obtained from Alfa Aesar (Ward Hill, MA). Carbon-coated Cu TEM grids, 400 mesh, were purchased from Electron Microscopy Sciences (Hatfield, PA). Disposable UV-transmissive cuvettes with a 1.0 cm path length were purchased from BRAND GmbH (Wertheim, Germany). All chemicals were used as received. Aqueous solutions were prepared using 18 MΩ·cm Milli-Q water (Millipore).

**Synthesis of AlkP and NP Solutions.** These solutions were prepared using a 1.0 M glycine solution adjusted to pH 9.2. Solutions were prepared in capped glass vials at an ambient temperature of 21 ± 2 °C. Four species were characterized in this work, and they are identified using the following notations: AlkP-Au@Ag₂O, AlkP-Au, and AlkP-Au@Ag₂O. AlkP solutions of enzyme alone were prepared by combining 4.0 μL of AlkP with 1.35 mL of the glycine solution, and then stirring for ~18 h. Au@Ag₂O, which is a NP species having a Au core and a Ag₂O shell (but no enzyme), was prepared by combining 1.0 mL of Au colloid solution and 0.35 mL of glycine, stirring for ~18 h, and then adding 50.0 μL of Ag⁺ solution. The mixture was then stirred for an additional 2.0 h. AlkP-Au, which is an enzyme-Au colloid conjugate, was prepared by combining 1.0 mL of Au colloid solution, 0.35 mL of glycine, 4.0 μL of AlkP, and then stirring for ~18 h. AlkP-Au@Ag₂O was prepared as described for AlkP-Au, with the additional steps of adding 50.0 μL of Ag⁺ solution followed by stirring for 2.0 h. All species were isolated by filtration in which the crude solution was transferred to the reservoir of a MWCO tube and centrifuged for 10.0 min at 3000 g. The filtrate was collected, and the pellet was resuspended with 1.0 mL of water and centrifuged again for 10.0 min at 3000 g. The eluted liquid, hereafter referred to as the wash, was pooled with the initial filtrate and retained for analysis. The pellet was resuspended with 1.0 mL of water and transferred to a microcentrifuge tube for subsequent analysis.

**Characterization.** UV–vis spectra of filtered and desalted solutions of Au colloid, Au@Ag₂O, AlkP-Au, and AlkP-Au@Ag₂O were obtained using a Hewlett-Packard HP8453 spectrometer (Santa Clara, CA). TEM micrographs of AlkP-Au, Au@Ag₂O, and AlkP-Au@Ag₂O were collected on a FEI Tecnai G2 F20 X-TWIN instrument (Hillsboro, OR). Grids were prepared by drop-casting 5.0 μL of each solution onto a TEM grid and drying overnight in a vacuum desiccator.

Element maps and scanning/transmission electron microscope (STEM) images of AlkP-Au@Ag₂O were captured on a Hitachi S5500 with a Bruker Quantax 4010 EDS detector operating at 30 kV in bright-field mode (Pleasanton, CA, and Billerica, MA, respectively). TEM grids for STEM and EDS were prepared as for TEM, but included an extra drying step where the grids were lyophilized for 48 h. XPS analysis was carried out using a Kratos Axis Ultra spectrometer having an Al Kα filament (Chestnut Ridge, NY). XPS spectra were collected for Au colloid, Au@Ag₂O, AlkP-Au, AlkP-Au@Ag₂O, and WT AlkP. Samples of Au colloid, Au@Ag₂O, AlkP-Au, and AlkP-Au@Ag₂O were prepared by drop-casting 20 μL volumes onto sections of Si wafer and drying for 24 h in a vacuum desiccator. WT AlkP specimens were prepared by drop-casting 10 μL of AlkP solution onto Au-coated glass and drying for 24 h in a vacuum desiccator. Spectra were collected at a pass energy of 20 eV with a step size of 0.1 eV for individual element scans. Sample charging was compensated by referencing the observed C 1s C–C peak to 284.5 eV.

**AlkP Activity Assay.** Time-resolved colorimetric assays were used to measure the active quantity of AlkP in diethanolamine (DEA) units (U) for WT AlkP, AlkP-Au, Au@Ag₂O, and AlkP-Au@Ag₂O. One AlkP DEA U is defined as the amount of enzyme required to hydrolyze 1 nmol of pNPP per minute. Activity testing was performed for each sample on the pooled filtrate and wash fraction, and on the resuspended pellet fraction. The activity assay method was adapted from literature reports. Assays were carried out in a buffer containing 1.0 M DEA and 1.0 mM MgSO₄ adjusted to pH 9.8 with H₂SO₄. Briefly, 1.7 mL of buffer, 20.0 μL of the test solution, and 0.10 mL of 150 mM pNPP were added to a cuvette and mixed by repetitive pipetting. The absorbance at 405 nm (A₄0₅) was recorded at 0.50 min intervals for 6.0 min. A least-squares fit was applied to the linear data to obtain the change in A₄0₅ as a function of time (∆A₄0₅ min⁻¹). The activity in U was then calculated from the ∆A₄0₅ min⁻¹ value as described in the literature.

Between 3 and 5 replicates for each analyte were assayed. Inhibition assays were performed on solutions of WT AlkP, AlkP-Au, and AlkP-Au@Ag₂O. For these assays, either urea or Phe was added to the DEA solution prior to the test. AlkP activity was measured at urea concentrations of 2.5 or 5.0 M and Phe concentrations of 10.0 or 50.0 mM; the UV–vis blank contained DEA buffer, inhibitor at an appropriate concentration, and pNPP. The inhibitor assays were performed using the same procedure as the activity assays, with the added step of allowing the test solution and inhibitor-containing DEA solution to incubate for 30.0 min prior to pNPP addition and UV–vis analysis. Different concentrations of enzyme were necessarily used for the WT AlkP, AlkP-Au, and AlkP-Au@Ag₂O assays, and therefore, the results were normalized as discussed in the Supporting Information.

**RESULTS AND DISCUSSION**

As discussed in detail in the Experimental Section, AlkP-Au@Ag₂O colloids were prepared in three steps. First, AlkP was mixed with citrate-capped Au colloid and glycine solutions. Glycine is compatible with AlkP activity without adversely affecting the Au colloids. Second, a Ag⁺ solution was added to this mixture. After incubation, the resulting material was purified and characterized. Additional information about the synthesis of the other NP species discussed in this report is provided in the Experimental Section.

TEM micrographs of Au@Ag₂O and AlkP-Au@Ag₂O are shown in Figure 1. In Figure 1a, two populations of Au@Ag₂O particles are evident: discrete particles and small aggregates. The core@shell nature of these materials is suggested by the contrast difference between the dark centers and lighter periphery. The contrast difference is more apparent in the higher magnification image provided in the inset. In Figure 1b, two populations of
AlkP-Au@Ag₂O are also observed: small spherical particles and larger agglomerates. The smaller particles resemble the Au@Ag₂O NPs in Frame a. The larger clusters were observed consistently in AlkP-Au@Ag₂O preparations, but they were not present in Au@Ag₂O samples. The inset of Figure 1b shows a high magnification image of an AlkP-Au@Ag₂O agglomerate, and it is apparent that these materials also have a core@shell structure. TEM analysis of AlkP-Au (Supporting Information, Figure S1) indicates well-dispersed, unagglomerated particles exhibiting uniform contrast. On the basis of the morphology of the large AlkP-Au@Ag₂O agglomerates and their presence only in the AlkP-Au@Ag₂O images, we draw two conclusions. First, the large AlkP-Au@Ag₂O agglomerates have a core@shell structure. Second, the formation of large agglomerates depends on the presence of AlkP.

UV–vis spectra for desalted, aqueous solutions of Au colloids, AlkP-Au, Au@Ag₂O, and AlkP-Au@Ag₂O are shown in Figure 2. Each spectrum was normalized to a maximum absorbance of 1.0 to facilitate comparison of the spectra. The Au colloid starting material has a peak centered at 523 nm, which corresponds to the plasmon reported for Au NPs in this size range. The solution containing AlkP-Au is similar to that of the Au colloid solution except for a slight shift in the plasmon band to 527 nm.

The spectrum of Au@Ag₂O reveals the effect of Ag deposition onto the Au colloids. In this case, a new peak is observed at 410 nm, but the Au plasmon previously observed in the spectra of the Au colloid and AlkP-Au solutions is absent. The position of the new peak arising from Au@Ag₂O corresponds to a Ag plasmon, which has previously been reported in the literature for Ag colloids, Ag₂O thin films, and Ag NPs having a Ag₂O shell (Ag@Ag₂O). The position of the peak observed for Au@Ag₂O is also similar to the plasmon band reported for bimetallic Au@Ag nanoparticles, in which the Ag plasmon is quenched by the presence of the Ag (or Ag₂O) shell. Accordingly, this finding is consistent with the core@shell structure revealed by the TEM images in Figure 1a.

The spectrum of AlkP-Au@Ag₂O is different than the other three spectra in that it exhibits two overlapping peaks at 427 and 505 nm. The position of the 427 nm peak is consistent with Ag₂O. However, the presence of the peak at 505 nm suggests that some fraction of the Au surface, probably in the larger agglomerates (Figure 1b), is not covered with Ag₂O. This view is consistent with results reported by Gonzalez et al., who observed a similar spectrum for bimetallic Au@Ag nanoparticles having only a partial Ag shell. Because protein adhesion to Au NPs is well documented, we speculate that AlkP is responsible for incomplete Ag₂O coverage and hence the band at 505 nm.

**XPS Analysis.** XPS spectra collected for Au colloids, Au@Ag₂O, AlkP-Au@Ag₂O, and WT AlkP are provided in Figure 3. The high-resolution C spectra in Figure 3a exhibit binding energies consistent with those reported for C–C, C–N, and C–O bonds. For example, the C 1s spectrum for Au@Ag₂O consists of two peaks having binding energies corresponding to C–C and C–O bonds. The presence of a C–O peak can be attributed to the citrate capping agent on the Au colloids. The spectrum of AlkP-Au@Ag₂O exhibits three dominant features. Two of these peaks have energies coincident with the C–C and C–O bonds in the Au@Ag₂O spectrum. The binding energy of the center peak in the AlkP-Au@Ag₂O spectrum is suggestive of
C—N bonding. These three features also appear in WT AlkP. Recalling that the AlkP-Au@Ag₂O solution was washed and filtered to remove free enzyme prior to XPS analysis, we conclude that AlkP is entrapped within these Au@Ag₂O agglomerates. The high-resolution Ag 3d XPS spectra of Au@Ag₂O and AlkP-Au@Ag₂O are provided in Figure 3b. Dashed lines indicate the reported binding energies for zerovalent Ag (368.3 and 374.3 eV).10,22 The 3d 5/2 peaks for Au@Ag₂O and AlkP-Au@Ag₂O appear at 368.1 and 367.7 eV, respectively, which are somewhat lower than the corresponding literature value for zerovalent Ag.10 However, these binding energies are consistent with values reported for Ag₂O (between 367.5 and 368.2 eV).10,15,23 That Ag₂O has a more negative binding energy than zerovalent Ag is attributed to the electronic structure of Ag₂O and its metal-like conductivity.24,25 We conclude that Ag₂O is the principal Ag species present in both Au@Ag₂O and AlkP-Au@Ag₂O. Ag₂O may form via reduction of Ag⁺ to Ag⁰ by citrate,26 which is used to stabilize the Au colloids, followed by reaction with oxygen to yield Ag₂O. High-resolution XPS spectra in the Au region are provided in Supporting Information Figure S2 and indicate the presence of zerovalent Au only.

**Figure 3.** (a) High-resolution C 1s XPS spectra for Au@Ag₂O, AlkP-Au@Ag₂O, and WT AlkP. The three features correspond to C—C (284.5 eV), C—N (286.0 eV), and C—O (287.5 eV) functional groups. (b) Ag 3d XPS spectra for Au@Ag₂O and AlkP-Au@Ag₂O. The dashed lines represent the 3d 5/2 and 3d 3/2 reference energies for Ag⁰.

**Figure 4.** EDS element map of an AlkP-Au@Ag₂O agglomerate and smaller, nearby Au@Ag₂O particles. The image is rendered in false color: Ag is shown in blue and Au in red. The inset is an STEM image of the EDS field-of-view.

**EDS Mapping.** We used element mapping to carry out spatially resolved analysis of the large AlkP-Au@Ag₂O agglomerates shown in Figure 1b. Figure 4 is a drift-corrected element map, rendered in false color, of a single cluster surrounded by several smaller particles. The inset is an STEM image of the EDS field of view. The map reveals two interesting features. First, the elemental distribution in the large cluster is consistent with that of a Au@Ag₂O core@shell morphology. Second, its Au core is much larger than the ∼5 nm colloids, which were the initial source of Au. This indicates the presence of multiple Au colloids in the core.

**Activity Assays and Inhibition.** Colorimetric activity assays were used to quantify the amount of AlkP present during each step of the synthesis of AlkP-Au@Ag₂O. The DEA assay described in the Experimental Section was selected to determine the AlkP concentration in activity units, because it reports an absolute quantity of the functional enzyme. As discussed earlier, the metal-containing solutions were purified to ensure that free AlkP was removed. This step is important for interpreting the activity assay, because it ensures that the measured activity arises only from AlkP associated with agglomerates. Filtration and washing steps were used for purification, and they result in three fractions. The initial filtrate consists of unbound AlkP, solvent, glycine, and salts that were introduced during the synthesis. The wash solution was used to ensure removal of free AlkP from the retained solid material comprises the second fraction. Finally, the solid remaining after the wash step was taken up in water, and this represents the third fraction. The filtrate and wash fractions were pooled and analyzed for enzyme activity alongside the resuspended solid for each of the following samples: WT AlkP, Au@Ag₂O, AlkP-Au, and AlkP-Au@Ag₂O. Information about the analysis of these data is provided in the Experimental Section.

The results from the activity assays, in terms of catalytically active enzyme units (U), are presented in Table 1. For WT AlkP, we find that essentially all enzyme activity arises from the pooled filtrate and wash, which is consistent with expectations for filtration of a 60 kDa molecular weight enzyme through a 100 kDa MWCO filter. Also consistent with expectations, the enzyme-free Au@Ag₂O particles registered effectively zero activity.

The effect of NPs on the activity of the enzyme is revealed by the results of the AlkP-Au assay. Here, there is a decrease in the total enzyme activity compared to free WT AlkP, which is typical for enzymes adsorbed to metal surfaces.27 Specifically, the total enzyme activity for the AlkP-Au material (pellet, filtrate, and...
Table 1. Active Quantity of AlkP in DEA Units (U) for the WT AlkP, Au@Ag₂O, AlkP-Au, and AlkP-Au@Ag₂O Species by Fraction

<table>
<thead>
<tr>
<th>species</th>
<th>filtrate + wash activity (U)</th>
<th>pellet activity (U)</th>
<th>total activity (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT AlkP</td>
<td>1.65 ± 0.07</td>
<td>0.022 ± 0.003</td>
<td>1.67 ± 0.07</td>
</tr>
<tr>
<td>Au@Ag₂O</td>
<td>0.00 ± 0.06</td>
<td>0.030 ± 0.004</td>
<td>0.030 ± 0.05</td>
</tr>
<tr>
<td>AlkP-Au</td>
<td>0.49 ± 0.07</td>
<td>0.87 ± 0.11</td>
<td>1.37 ± 0.13</td>
</tr>
<tr>
<td>AlkP-Au@Ag₂O</td>
<td>0.79 ± 0.05</td>
<td>0.21 ± 0.01</td>
<td>1.00 ± 0.05</td>
</tr>
</tbody>
</table>

Activities were measured for the pooled filtrate plus wash fractions and the resuspended pellet. The total activity is shown in the column on the right. The values shown represent the average of 3–5 replicates carried out using independently prepared materials (±1σ).

Summary and Conclusions

We have described a means for stabilizing AlkP against two inhibitors: urea and Phe, which operate on the enzyme by different mechanisms. Structural characterization suggests that the enzyme is incorporated into a conductive Au@Ag₂O core-shell structure, but beyond this, we are not prepared to discuss the molecular-level details that lead to stabilization. There are two aspects of the findings reported here that are significant. First, the stabilizing agent is electrically conductive, and therefore there is a chance that this approach, or one like it, could be used to stabilize redox enzymes and electrically wire them to an electrode surface. Second, the time required to prepare the AlkP-Au@Ag₂O construct is one day. This is significantly faster than has been reported previously for sol–gel and other types of enzyme stabilizers.

Associated Content

Supporting Information. TEM micrographs of AlkP-Au, Au 4f XPS spectra, and an explanation of how the results presented in Figure 5 were calculated. This material is available free of charge via the Internet at http://pubs.acs.org.

Acknowledgment

We gratefully acknowledge financial support from the National Science Foundation (Grant No. 0847957), and the Robert A. Welch Foundation (Grant No. F-0032). The NSF also provided support for the X-ray photoelectron spectrometer used in this study (Grant No. 0618242). We gratefully acknowledge Dr. Domingo Ferrer (Microelectronics Research Center, UT-Austin) for help with the TEM measurements.

References

Langmuir


