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Coimmobilization of Acetylcholinesterase and Choline Oxidase on Gold Nanoparticles: Stoichiometry, Activity, and Reaction Efficiency

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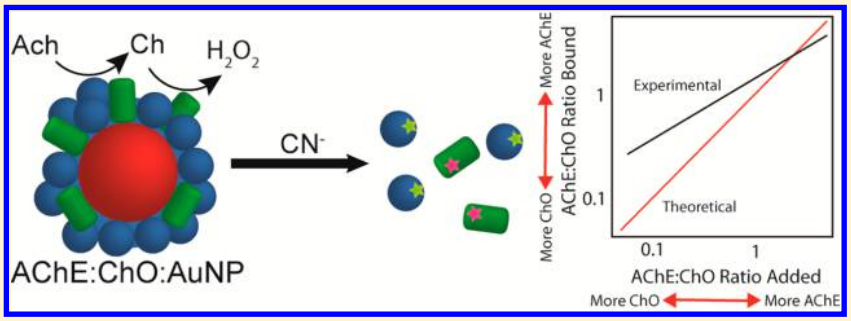
Supporting Information



ABSTRACT: Hybrid structures constructed from biomolecules and nanomaterials have been used in catalysis and bioanalytical applications. In the design of many chemically selective biosensors, enzymes conjugated to nanoparticles or carbon nanotubes have been used in functionalization of the sensor surface for enhancement of the biosensor functionality and sensitivity. The conditions for the enzyme:nanomaterial conjugation should be optimized to retain maximal enzyme activity, and biosensor effectiveness. This is important as the tertiary structure of the enzyme is often altered when immobilized and can significantly alter the enzyme catalytic activity. Here we show that characterization of a two-enzyme:gold nanoparticle (AuNP) conjugate stoichiometry and activity can be used to gauge the effectiveness of acetylcholine detection by acetylcholine esterase (AChE) and choline oxidase (ChO). This was done by using an analytical approach to quantify the number of enzymes bound per AuNP and monitor the retained enzyme activity after the enzyme:AuNP synthesis. We found that the amount of immobilized enzymes differs from what would be expected from bulk solution chemistry. This analysis was further used to determine the optimal ratio of AChE:ChO added at synthesis to achieve optimum sequential enzyme activity for the enzyme:AuNP conjugates, and reaction efficiencies of greater than 70%. We here show that the knowledge of the conjugate stoichiometry and retained enzyme activity can lead to more efficient detection of acetylcholine by controlling the AChE:ChO ratio bound to the gold nanoparticle material. This approach of optimizing enzyme gold nanoparticle conjugates should be of great importance in the architecture of enzyme nanoparticle based biosensors to retain optimal sensor sensitivity.

Enzyme:gold nanoparticle conjugates are employed for a variety of catalytic functions, due to their small size, ease of preparation, and recoverability. The enzymatic and stoichiometric characterization of enzyme:gold nanoparticle (AuNP) conjugates has proven to be a useful tool in creating nanoparticle based detection methods. Previous work in the area has shown that assumptions based on enzyme properties in solution, such as charge and size, cannot be used to predict the amount or activity of an enzyme immobilized onto a nanoparticle surface of almost any kind,[1−5] and that controlling parameters such as enzyme:AuNP stoichiometry and multilayer effects can have a large impact on conjugate activity.[1,2,6,7] Colocalization and coimmobilization of enzymes have been reported for combinations of two[2,8] and three[6] enzymes and have demonstrated increased sequential catalysis, and the importance of enzyme organization in multilayer conjugates.

The reaction efficiency for a multienzyme system is an important characteristic for the design and use of enzyme:AuNP conjugates. In solution, multienzyme reactions are designed so that the enzymes responsible for the downstream reactions are in excess and do not limit the sequential rate. Ideally, when enzymes are in close proximity, the efficiency of producing the final product is high, because placing the enzymes in close proximity increases the reaction efficiency by limiting the possibility of substrate diffusion away from the reactive sites, and decreases the time needed for the sequential reaction. Assuming that all substrates and intermediates are effectively catalyzed into the final product can cause misleading data interpretation, especially when a quantitative measure is desired. Additionally, the concentration of substrate and the buildup of intermediates, which can affect the reaction rates, must be accounted for. By characterizing the reaction efficiency, defined for a two-enzyme system as the quotient of the sequential activity and the activity of the first enzyme (eq 1), an accurate determination of the reliability of the conjugate can be

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made. Previously, Pescador et al. defined the reaction efficiency for immobilized enzyme in a similar way. In 2008, they found a slightly greater than 40% efficiency for glucose oxidase and horseradish peroxidase with the intermediate hydrogen peroxide.[8]

$$\% \text{ efficiency} = \frac{v_{seq}}{v_{Ach}} \times 100 \quad (1)$$

Enzyme based detection methods have become a common tool for detecting and quantifying substances that are difficult or impossible to measure through standard analytical techniques alone, especially when a short time scale of seconds or milliseconds is required.[9−12] Acetylcholine is an important neurotransmitter in the mammalian nervous system, which is difficult to quantify through colorimetric and electrochemical means. Current acetylcholine sensors that have been developed have a response time on the order of seconds and hence are too slow for recordings for neurochemical release that occurs on the time scale of milliseconds. Therefore, to be able to monitor acetylcholine release at single cells or in the brain, a fast, quantitative, and sensitive small-scale method for detecting acetylcholine is sorely needed. The sequential catalytic reaction of the two enzymes acetylcholinesterase (AChE) and choline oxidase (ChO) have been employed to create several colorimetric and electrochemical biosensors based on the conversion of acetylcholine to a luminescent, colorimetric, or electrochemically active product.[9,11−24,43] Many of these methods use gold nanoparticles and enzyme immobilization to create stable sensors, for which the characterization of enzyme nanoparticle stoichiometry, enzyme activity, and reaction efficiency can aid in the design and optimization of a sensor.

Our motivation for this work is to use analytical methods to characterize and thereby optimize the enzyme catalytic efficiency of conjugates created with both AChE and ChO. This will allow us to develop a reliable scheme for acetylcholine detection by maximizing the conversion of acetylcholine to a detectable product and indicating the limitations of the method. This analysis is the first step to analytically determine the optimal synthesis conditions for a two-enzyme nanoparticle conjugate system that can serve as essential building blocks in the development of a fast, sensitive, and selective method for acetylcholine detection at single secretory cells and in vivo in the brain. The optimization of nanoparticle conjugates with colorimetric readout will allow us to quickly and efficiently characterize enzyme adsorption and activity, allowing us to build upon this knowledge as the conjugates are implemented with electrochemical detection in the next steps.

■ RESULTS AND DISCUSSION

In this work, we characterize and study two enzymes acetylcholinesterase (AChE) and choline oxidase (ChO) immobilized to gold nanoparticles (AuNP) in order to determine an optimal configuration for the detection of the neurotransmitter acetylcholine, through the catalytic conversion to hydrogen peroxide, which is an electroactive molecule that can easily be detected at the surface of an electrode. Three types of conjugates were created as seen in Figure 1. To study the basic interactions of each enzyme with the gold surface, the first two conjugates containing one enzyme species, either AChE or ChO, were first created. Second, conjugates containing both enzyme species were created with various ratios of AChE:ChO to further characterize their joint interactions with the gold surface as well as each other.

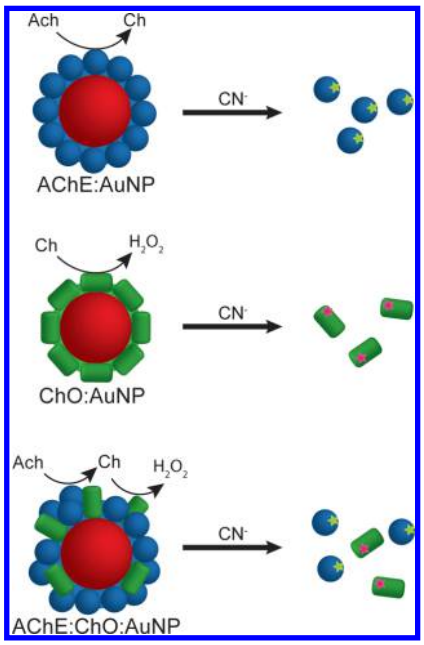

Figure 1. Enzyme:AuNP conjugates were created by direct adsorption to the gold nanoparticle surface by one or both enzymes present in bulk solution during synthesis. To quantify the number of enzymes bound to a AuNP, potassium cyanide was used to remove the AuNP core and allow for quantification of fluorescently tagged enzymes.

For all experiments, gold nanoparticles created by citrate reduction were functionalized with AChE and/or ChO through direct immobilization. As seen in Supporting Information Figure S-1, the absolute nanoparticle diameter was determined by transmission electron microscopy (TEM) to be 14 ± 1 nm (n = 152). This was confirmed through dynamic light scattering (DLS) by the presence of a single population with a hydrodynamic diameter of 18 ± 2 nm (Supporting Information Figure S-2) and the presence of a UV−vis adsorption peak at 519 nm (Supporting Information Figure S-3), both indicative of the presence of nanoparticles of this size. A total ratio of 100:1 enzyme:AuNP was chosen to control the amount of enzyme adsorbed per AuNP. This low ratio was used to prevent multilayer coverage, which may hinder efficient acetylcholine conversion. Hence, our goal was to design and characterize an enzyme gold nanoparticle conjugate with monolayer coverage of enzymes.

Characterization of AChE:AuNP and ChO:AuNP Conjugates. To independently study the affinity of AChE and ChO on the surface of AuNP, conjugates of AChE:AuNP and ChO:AuNP were created, as seen in the top and middle panels of Figure 1. Measurements of conjugate stoichiometry were made to determine how much enzyme was present per nanoparticle, to elucidate if the AuNPs were completely coated and if more than monolayer coverage was found. The amount of enzyme present in conjunction with the activity of each enzyme allowed for the determination of specific activity for each enzyme, which is an excellent indication of the catalytic ability and retained structure after immobilization.

In some cases, enzyme monolayer coverage and conjugate stoichiometry can be estimated by knowing the surface area of the AuNP and area an individual enzyme molecule would occupy, called the footprint.[1,2,44,45] The molecular weight and

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crystal structures of each enzyme was used to determine the footprint size and predict conjugate stoichiometry, the ratio of enzyme:AuNP, for each enzyme. As seen in Table 1, AChE and ChO are very similar in size and determined by their molecular weights. From this information, it would be expected for AChE and ChO to form monolayer coverage on AuNP at similar ratios. However, from examination of the crystal structures it is evident that while AChE is spherical[25] in shape, ChO is cylindrical;[26] this leads to a greater variability in the predicted adsorption ratio for each enzyme.

Table 1. Characterization of Single Enzyme:AuNP Conjugates

	AChE	ChO
molecular weight	67 000 Da	66 000 Da
isoelectric point	5.5	4.1
predicted stoichiometry	27:1	19:1
stoichiometry found	28 (± 2):1	15.2 (± 0.8):1
specific activity (free enzyme)	1000 U/mg	10 U/mg
specific activity (conjugate)	125 ± 18 U/mg	10.1 ± 0.9 U/mg
activity by area	63 ± 5 mU/cm^2	2.7 ± 0.3 mU/cm^2

Conjugate stoichiometry was experimentally determined with fluorescently tagged enzymes, AlexaFluor488 tagged AChE and AlexaFluor555 tagged ChO, using previously established methods.[1,2] As expected from the predicted ratios, the enzyme:AuNP ratios for AChE and ChO are quite different. The footprint of AChE estimated a 27:1 enzyme AuNP ratio in comparison to 19:1 for ChO onto the AuNP surface. Notably, the predicted and experimentally determined ratios are quite similar, confirming that only about a monolayer of enzyme is present on the surface of each conjugate. For many enzymes, the determined conjugate stoichiometry is significantly higher than what would be predicted for monolayer coverage.[1,2] As previously shown by the Keating group,[1,2] the amount of enzyme adsorbed to a AuNP surface can be a function of the ratio of enzyme:AuNP present in solution during the conjugation process, where higher ratios result in multiple layers of enzyme being adsorbed per nanoparticle. Since this phenomenon was also shown to reduce the overall activity of enzyme conjugates in these experiments, the enzyme:AuNP ratios in this work were intentionally kept low to prevent multilayer formation.[1,2,27−29]

Specific activity, defined as the enzymatic activity in μmol/min divided by the amount of enzyme present in mg, is a measure of enzyme function after immobilization. Activity measurements for AchE:AuNP with acetylcholine and ChO:AuNP with choline were conducted and compared to the activity reported for the enzymes in bulk solution as provided. By using colometric readout for these experiments we can easily compare our results to the standard free enzyme assays conducted under similar conditions. As seen in Table 1, AChE and ChO each respond differently when immobilized. Adsorbed AChE loses nearly 90% of its activity due to the interactions with the AuNP surface, while ChO activity does not change significantly. Throughout the literature, changes in enzyme activity, both increases and decreases, have been reported after immobilization to a surface.[2−4,28,30−37] These changes are enzyme specific, and in most of these reports, the changes in the activity are linked to denaturation of the protein structure and changes in the active site configuration, steric hindrances, and active site blockage preventing proper catalysis.[1,2,6] The presence of multiple layers of enzyme on the surface of the AuNP has also been linked to reductions in activity.[1]

The activity per nanoparticle, or per square centimeter, is another important measure of the catalytic ability of an enzyme conjugate, which is a function of not only the specific activity of an enzyme, but also the particle stoichiometry. From the data presented in Table 1, it is evident AChE is about 20x more catalytically active than ChO after immobilization. With this information it can be predicted that a low AChE:ChO ratio should be optimal for acetylcholine detection through the production of hydrogen peroxide, since the greatest reaction efficiency for the sequential enzymatic reaction would be achieved when there is more ChO activity than AChE activity.

Characterization of AChE:ChO:AuNP Conjugates. To allow for a sequential reaction to occur between AChE and ChO, conjugates were prepared with a combination of both enzymes present. As demonstrated with the AChE:AuNP and ChO:AuNP conjugates, a low total enzyme:AuNP ratio leads to monolayer enzyme coverage. Additionally, due to the higher catalytic activity of AChE, low AChE:ChO ratios may lead to a greater overall reaction efficiency per nanoparticle, which is essential for the effective detection of acetylcholine enzymatically. Therefore, in these studies, the total enzyme:AuNP ratio was held to 100:1, and enzyme:enzyme ratios from 5:1 to 1:20 of AChE:ChO per AuNP were tested in order to characterize how the enzymes work in combination as well as their immobilization stoichiometry and individual enzymatic activities.

As seen in Figure 2A, as the ratio of AChE:ChO immobilized to AuNP is varied and the total enzyme:Au ratio held to 100:1, the enzyme:AuNP stoichiometry of each enzyme is altered significantly. When a high enough concentration of either enzyme is present in solution (AChE at high ratios and ChO at low ratios), the conjugate appears to saturate with that enzyme and no further significant change is seen. Saturation is evidenced by the deviation from the overall trend, and for AChE it can be found for ratios higher than 2:1 AChE:ChO, and for ChO at ratios lower than 1:5 AChE:ChO. In each of these occurrences, the AuNP may have become saturated by the amount of enzyme adsorbed, as slightly greater than monolayer amounts of AChE are present above 2:1 and ChO below 1:5.

At each ratio, the total amount of enzyme:AuNP slightly exceeds what would be necessary for monolayer formation, but is significantly less than what would be necessary for multiple layer formation. This could indicate that AChE and ChO have a slight affinity for each other, or that ChO is oriented differently as its cylindrical shape provides an alternative way for the enzyme to adsorb to the AuNP surface, with a different footprint size for each side. Similar results were found previously for malate dehydrogenase (MDH) and citrate synthase (CS), where an interaction between the two enzymes allowed for multiple layers of enzyme to be added to the same nanoparticle in multiple organizations.[2]

By comparing the AChE:AuNP and ChO:AuNP ratios found in Figure 2A, the AChE:ChO ratio bound per AuNP can be obtained and compared to the ratio added in solution, as presented in Figure 2B. In all cases, the ratio bound is significantly different from the ratio added. At the 2:1 and 5:1 AChE:ChO ratio added, the ratio bound is 2.4:1 to 3:1, respectively, and the values are not significantly different. Similarly, at the 1:10 and 1:20 AChE:ChO ratio added, the

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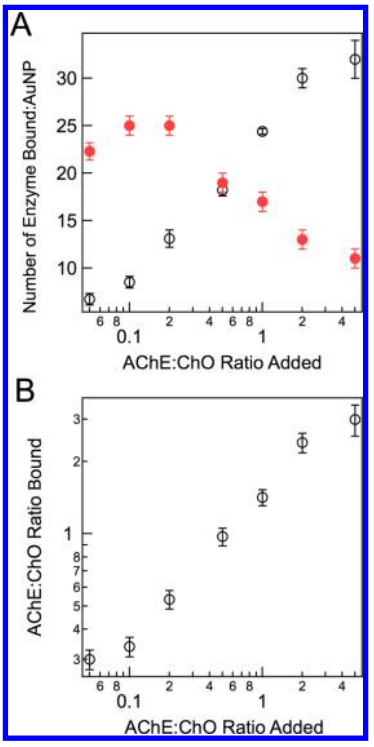

Figure 2. Determination of the stoichiometry of AChE versus ChO bound per AuNP, with varying ratios of AChE:ChO added in bulk solution during the conjugation process. The total enzyme:AuNP ratio was held constant to 100:1 in all measurements. (A) The amount of each enzyme adsorbed per AuNP was determined by using Alexa-488 tagged AChE (black, open symbols) and Alexa-555 tagged ChO (red, filled symbols). (B) From this information, the AChE:ChO ratio bound per AuNP could be determined. Error reported as standard error of the mean, $n = 5-10$.

ratio bound does not change significantly. This is likely due to the saturation of the AuNP surface with AChE at high ratios and ChO at low ratios.

At ratios of 1:1 to 1:5 AChE:ChO added, the ratio bound is approximately 1.5−3 times greater than the ratio added. This may indicate that AChE has a higher affinity for the AuNP surface than ChO, and outcompetes ChO to bind on the gold surface, and that the ratio bound cannot be assumed to be equivalent to the ratio added. Again, similar results have been found for MDH and CS when coimmobilized, where when equal amounts of MDH and CS are present in solution, more MDH adsorbs than CS.[2] Without a difference in a pronounced physical characteristic such as surface charge or hydrophobicity, or size, it is difficult to hypothesize which enzymes or proteins will have the greatest affinity for the nanoparticle surface, making direct quantification necessary to determine stoichiometry.

By quantifying the number of each enzyme bound to the surface of each AuNP after conjugation, the specific activity of each enzyme can be determined. Hence, to determine if the AChE:ChO ratio has an effect on the activity of either enzyme, the specific activity for both AChE and ChO was monitored for each AChE:ChO ratio. As seen in Figure 3, neither enzyme activity changes significantly with the AChE:ChO ratio, and when further compared to the AChE:AuNP and ChO:AuNP conjugates it is evident that enzyme activity is not significantly impacted by the presence of the second enzyme. In previous reports, it has been shown that the presence of a second

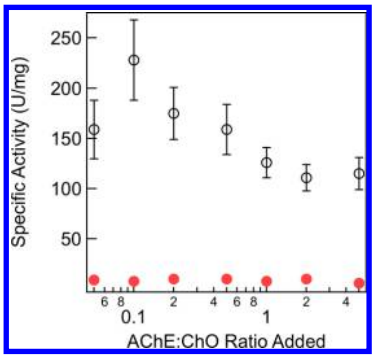

Figure 3. Specific activity of AChE (black, open symbols) and ChO (red, filled symbols) in AuNP conjugates created with varying ratios of AChE:ChO where the total enzyme:AuNP ratio was held to 100:1. Error reported as standard error of the mean, $n = 5-10$.

enzyme can have a drastic effect on enzyme activity. The presence of multilayers on the surface of the AuNP can cause losses of up to 5 times (caused by steric hindrances), and increases in efficiency of more than 10 times (caused by enzyme−enzyme as apposed to enzyme−nanoparticle interactions) have been found when enzymes are coimmobilized.[2] Since the formation of multilayers was intentionally avoided here, no significant difference between the specific activity of individually and coimmobilized conjugates is found.

While specific activity is a good indication of enzyme structure and function after immobilization, it is not indicative of the catalytic activity of the overall conjugate. By knowing the surface area of the nanoparticles, the activity per surface area gives a measure of the efficiency of the conjugate that can act as a sensor surface. To establish the catalytic properties of the AuNP conjugates, the activity per nanoparticle was determined for both AChE and ChO at each AChE:ChO ratio. Figure 4 shows how the activity per nanoparticle, expressed as activity per square centimeter, varies for both AChE (Figure 4A) and ChO (Figure 4B). These units were chosen so that comparisons to conjugates of different diameters or planar surfaces would be possible. For each enzyme, the activity appears to be a function of the amount of each enzyme present and increases with increasing stoichiometry, shown in Figure 2A. As could be expected, the AChE activity is greater than the ChO activity at each ratio due to the more than 10× difference in specific activity, shown in Figure 3. Thus, the total activity is not only determined by the amount of enzyme present, but also by how active the individual enzyme molecules remain after adsorption.

Sequential Activity of AChE and ChO. To effectively detect acetylcholine enzymatically, AChE and ChO must work sequentially to convert acetylcholine to choline and finally to betaine and hydrogen peroxide, the latter of which can be quantified by HRP activity as seen here or electrochemically as in the case of biosensors.[38] As seen in Figure 5, for each AChE:ChO ratio, the sequential activity is higher than the rate of ChO activity; this could be due to channeling of choline between AChE and ChO because they are in close proximity to each other, or a change in the AChE active sites that allow it to catalyze a product which can react with HRP.

Figure 6A shows the activity of AChE:AuNP (in the absence of any ChO) and ChO:AuNP (in the absence of AChE) with acetylcholine and choline as substrates. In their native conformations, no AChE activity can be detected without the presence of ChO (Figure 6B), while the AChE:AuNP activity

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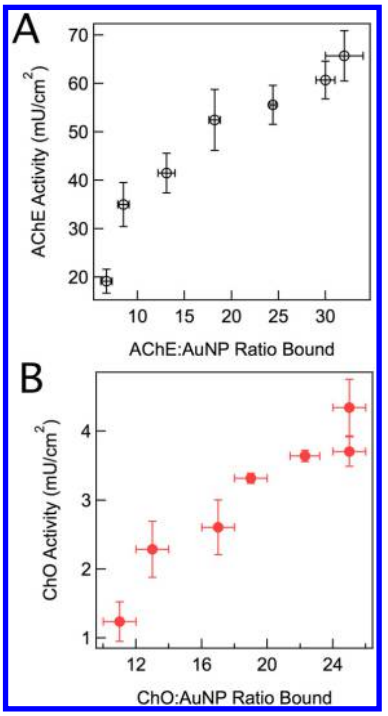

Figure 4. Activity of (A) AChE (open symbols) and (B) ChO (filled symbols) can be measured as a function of the AuNP surface area expressed in cm^2. Error reported as standard error of the mean, n = 5−10.

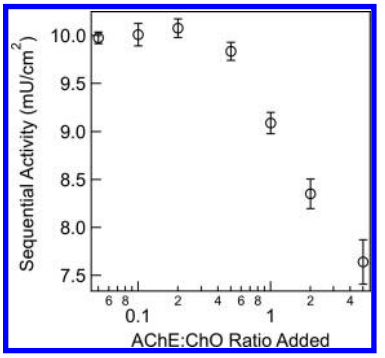

Figure 5. Sequential activity of AChE and ChO conjugates created with varying AChE:ChO ratios in which the total enzyme:AuNP ratio was held constant to 100:1. The sequential activity can be measured as a function of the area of the AuNP.

can be detected with both acetylcholine and choline. This may be due to significant changes in the structure of immobilized AChE, as evidenced by the almost 90% loss of activity found for the conjugate. Structural changes have previously been shown in immobilized AChE by Shin et al. in 1996 with liposomes[39] and Wang et al. in 2009 by a variety of nanoparticles.[30] This could deplete the specificity of AChE for acetylcholine and allows for other substrates to be catalyzed such as choline, betaine, and betaine aldehyde which are all steps in the production of hydrogen peroxide by ChO. Additionally, the presence of a reaction between ChO and acetylcholine may point to a similar, though less extensive, loss of specificity (Figure 6A).

The sequential activity follows a similar trend to ChO activity (Figure 4B), which indicates that ChO may be the limiting reaction in the sequential rate. Ideally, AChE activity should be the rate-limiting step so that all of the intermediate, choline,

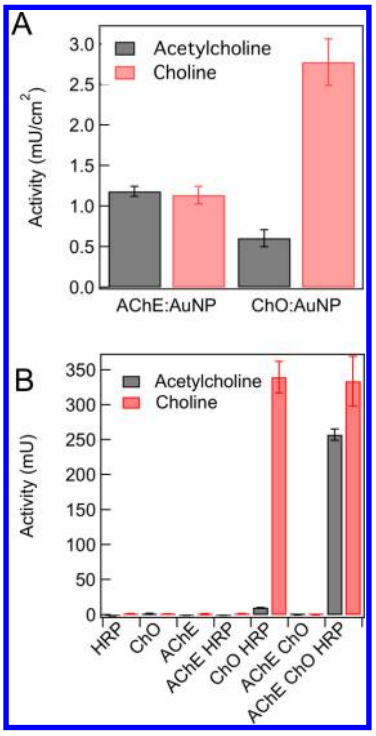

Figure 6. Conjugates were tested for selectivity in terms of analyte cross reactions in activity assays with each enzyme and carried out with all possible substrates. (A) Activity of both AChE:AuNP and ChO:AuNP was measured using acetylcholine (black bars) and choline (red bars). (B) Activity of native enzymes was measured using acetylcholine (black bars) and choline (red bars). Error reported as standard error of the mean, n = 5−10.

produced by AChE is quickly consumed and there is no opportunity for a buildup of intermediate. At high concentrations, this may lead to inhibition of either enzyme or diffusion away from ChO, which would lower the overall rate of catalysis. Since the sequential activity, similarly to the ChO activity, does not increase at ratios lower than 1:5 AChE:ChO, it is unlikely that a conjugate with greater sequential activity could be formed by altering the AChE:ChO ratio. Therefore, completely efficient detection of acetylcholine is unlikely, and knowledge of the efficiency and reliability the enzyme system is critical for effective exploitation of this method.

Reaction Efficiency. To determine how efficiently hydrogen peroxide production is from coimmobilized AChE and ChO, conjugates with a 1:10 AChE:ChO ratio were assayed for AChE and sequential activity with different concentrations of acetylcholine. As seen in Figure 7, the reaction efficiency, measured as the quotient of sequential activity and AChE activity, decreases with increasing acetylcholine concentration. This shows the dependence on reaction efficiency on the acetylcholine concentration, and indicates that not all the acetylcholine catalyzed by AChE to choline is catalyzed by ChO, likely because of diffusion away from conjugate in solution. As noted before, previous efficiency measurements for a two enzyme system have slightly greater than 40%.[8] The experiments reported here show an efficiency around 40% with high concentrations of acetylcholine, which increases with decreasing concentrations of acetylcholine. This is likely due to the diffusion of intermediates away from the active sites of the conjugate, which results in a lower amount of acetylcholine detected. Since 100% efficiency is not reached even at low

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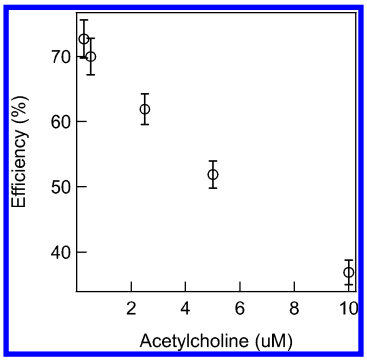

Figure 7. Reaction efficiency for the conversion of acetylcholine to hydrogen peroxide, determined for the sequential enzyme reaction measured for AChE:ChO:AuNP as determined by eq 1, with different concentrations of acetylcholine. Error reported as standard error of the mean, n = 5−10.

concentrations of acetylcholine, it is likely that many detection methods based on this and similar strategies are more qualitative than quantitative.

CONCLUSIONS

Here, we have shown that characterization of a two enzyme:AuNP conjugate stoichiometry and activity allows us to gauge the effectiveness of acetylcholine detection by AChE and ChO. From these experiments, it is evident that predictions are difficult to perform for a bienzyme nanoparticle conjugate using efficiency data of a single enzyme from bulk solution measurements. For instance, quantification of the enzymes bound to the AuNP surface shows that the ratio of AChE and ChO bound to the AuNP surface differs significantly from the enzyme ratio added in bulk solution during synthesis. This is most likely due to a difference in enzyme adsorption to the AuNP surface, where AChE seems to have a higher affinity to the AuNP surface than ChO. In addition, characterization of AChE:AuNP and ChO:AuNP conjugates showed that the enzymes are affected differently by the adsorption process to the AuNP surface. The AChE activity showed an 8-fold reduction in AChE activity in comparison to bulk solution, whereas the activity of ChO is not affected by the adsorption process to the AuNP surface.

It has been previously established that several factors including enzyme coverage affect the specific activity of enzymes. We therefore used specific activity measurements to optimize the AChE and ChO nanoparticle conjugation conditions by constraining the overall enzyme:AuNP ratio to monolayer enzyme coverage. Hence, the limiting of an enzymatic coverage to a monolayer can prevent loss of ChO catalysis due to a decrease in activity through steric and diffusional hindrances.

Therefore, the data from the assays of the specific enzyme activity for AChE and ChO allows for a better design of sequential enzyme conjugates, through stoichiometry and activity measurements.

From this careful analysis of quantifying the number of enzymes attached per AuNP and the resultant enzyme activity after the conjugation process, the AChE:ChO ratios employed allow for the optimization of acetylcholine detection. Finally, by defining and characterizing the efficiency of the sequential reaction, an often-overlooked parameter, we can optimize the design of future biosensors for the best possible sensitivity.

METHODS

Materials. AlexaFluor 488 and AlexaFluor 555 protein labeling kits were purchased from Invitrogen (Carlsbad, CA). AChE from electric eel, ChO from *Alcaligenes* sp., HRP type VI, acetylcholine chloride, choline chloride, *o*-phenylenediamine, hydrogen peroxide (30%), sodium citrate, tetrachloroaurate, sodium phosphate dibasic, potassium phosphate monobasic, sodium chloride, potassium chloride, potassium cyanide, and sodium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO). Whatman 20 nm pore diameter syringe filters were purchased from VWR. Lacey Formvar/carbon 300 mesh TEM grids were purchased from Caspilor, Sweden. Deionized water with resistivity ≥ 18 MΩ was used in all experiments.

Nanoparticle Synthesis. Gold nanoparticles were synthesized by the Turkevich method.[40] Briefly, 1 L of 1 mM $HAuCl_4$ was brought to a boil under stirring and reduced by the addition of 50 mL of 38.8 mM sodium citrate which was quickly added, and the solution allowed to boil for an additional 10 min before the heating source was removed. The nanoparticle diameter was determined to be 14 ±1 nm by TEM using a JEM-1200 EX II transmission electron microscope operated at 120 kV (JEOL, Tokyo, Japan), and the resulting images analyzed with ImageJ.[41]

Enzyme Labeling. AChE and ChO were resuspended in 5 mM sodium bicarbonate (pH 8.3) at a 2 mg/mL concentration for labeling. AChE and ChO were labeled with AlexaFluor488 and AlexaFluor555, respectively, according to the protocol provided by Invitrogen. Briefly, enzymes were labeled by incubation with succinimidyl-AlexaFluor dyes while stirring for 2 h. The resulting fluorophore−enzyme conjugates were then purified by size exclusion chromatography, and the concentration and degree of labeling were determined by UV−vis absorption using a Cary50 spectrophotometer.[42]

Conjugate Preparation and Purification. AChE:AuNP and ChO:AuNP were prepared at 100:1 enzyme:AuNP ratios by incubating 8.82 × 10^{-13} mol of AuNP; preparation and purification followed previously reported procedures.[2] Conjugates containing both AChE and ChO were created in a similar fashion where the appropriate ratio of AChE:ChO was added to solution; in all cases, the total enzyme:AuNP ratio was held to 100:1. In all cases, enzymes were allowed to adsorb to the surface of the nanoparticle for 30−60 min and excess enzyme was removed by centrifugation of the conjugates at 10 000g for 30 min performed three times.

Stoichiometry Determination. The amount of each enzyme present per AuNP was determined following a previously published method for the direct comparison of enzyme to nanoparticle concentrations.[2,45] Briefly, after washing away excess enzyme and measuring conjugate concentration by absorbance as described previously[1,2,45] with a Cary50 spectrophotometer, an aliquot (100 μL) of conjugate was dissolved in 20 mM KCN in phosphate buffered saline (PBS) overnight. A Horiba Jobin Yvon Fluorolog fluorimeter was then used to determine the concentration of each fluorescently labeled enzyme in solution. Stoichiometry is stated as the ratio of enzyme to AuNP determined by the concentration of each.

Activity Assays. All activity assays were conducted at room temperature in PBS buffer using coupled reactions, where an excess of reporter enzyme (HRP) is used to facilitate detection without limiting the reaction rate. Briefly, for ChO, activity was measured using 5 μM choline to produce hydrogen peroxide which was detected colorimetrically by coupling the reaction to HRP, which utilizes hydrogen peroxide to oxidize with 10 μM *o*-phenylenediamine. HCl was used to quench the reaction, and the product was quantified by the absorption at 492 nm using an extinction coefficient of 550 cm^{-1} M^{-1} for 2,3-diaminophenazine in PBS using a Cary50 spectrophotometer. AchE activity was measured similarly using 5 μM acetylcholine as the reactant with ChO and HRP as coupled reactions. The contribution of AuNP to the absorbance at 492 nm in all measurements was accounted for by including the same concentration of AuNP in blank assays.

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■ ASSOCIATED CONTENT

Supporting Information

Nanoparticle characterization data, including TEM, DLS, and UV−vis measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript

Notes

The authors declare no competing financial interest.

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