THE JOURNAL OF PHYSICAL CHEMISTRY B

Article

Cite This: J. Phys. Chem. B 2019, 123, 3962−3967

pubs.acs.org/JPCB

Multienzyme, Multistep Biosensor Produced through Kinetic Doping

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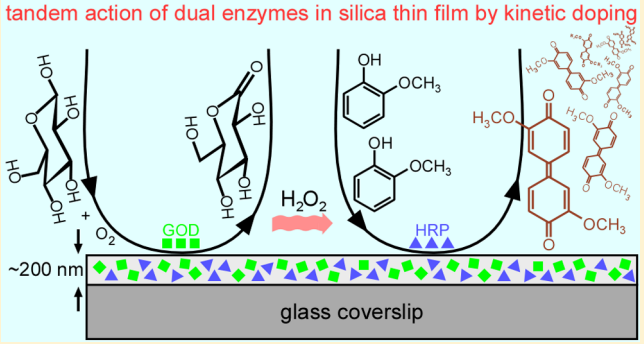
Supporting Information

ABSTRACT: The recently developed kinetic doping technique has shown promise in loading individual enzymes for use as a biosensor. In this study, the first example of kinetic doping to produce a biosensor loaded with more than one enzyme and using a multistep reaction pathway for detection is presented. Glucose oxidase (GOD) is shown to load both individually and together with horseradish peroxidase (HRP) with the tandem action of the two enzymes proving to be effective at detecting glucose in solution. Using a calculation based on known maximum loadings and experimentally determined activities, the final dual-enzyme thin films of known volume are shown to contain 1.8 ± 0.1 mmol/L of HRP and 0.22 ± 0.01 mmol/L of GOD, which represent 33 and 92% of loading efficiencies that each enzyme is known to be, respectively, capable of in a singularly loaded thin film. With the high loading afforded by the kinetic doping process under benign conditions, the thin films are able to load both enzymes all at once in an amount sufficient to function as an efficient biosensor. The most advantageous aspects of this process are its ease of production, involving only a few steps to produce highly loaded thin films that require no additional processing to function as intended, as well as the protein friendly environment that exists in the sol−gel film at the time of enzyme loading. This removes many typical restrictions on immobilizing protein and opens up a wider range of enzymes amenable to the process that enables the fabrication of more complex multistep biosensors utilizing a large array of proteins in the foreseeable future.



INTRODUCTION

Biosensors and their improvement have long been a topic drawing considerable amounts of interest from the scientific community.[1−6] Methods and techniques to transition from the harder to work with aqueous enzyme solutions to more convenient solid-state systems without losing the function of the enzyme have long been and continue to be an area of ongoing investigation.[7−12] Among these methods, one that has drawn particular attention is using silica sol−gel to form a solid matrix either encapsulating the enzyme or providing a surface for the enzyme to adhere to.[13−16]

Kinetic doping, a recent development in incorporating an enzyme into the silica sol−gel matrix, was utilized to produce thin films highly loaded with the proteins horseradish peroxidase (HRP) or cytochrome *C* (CyC) via spin coating and dip coating.[17−19] This technique proved more viable in loading enzyme than many conventional approaches, such as pre- or postdoping, while retaining a simplistic processing method attractive to many potential applications.[18,19] Furthermore, the kinetic doping method provides an enzyme friendly doping environment largely free of ethanol usually present in a sol−gel process, thereby eliminating any additional processing steps aiming at suppressing the effect of alcohol at the point where the enzyme is introduced.[19]

This enzyme friendly environment alongside the high loading of kinetic doping[18] and the ease of the production process presents a unique opportunity to incorporate not just a single protein into the thin film but multiple proteins capable of working together in tandem to offer a more elaborated sensing scheme while constrained together within a single thin-film-coated substrate. With the increased flexibility in types of surfaces and surface area that can be coated and kinetically doped, the inclusion of multiple enzymes need not reduce the activity of each below the point where the biosensor becomes impractical for regular use.

In this work, the kinetic doping technique is applied for the first time to build multienzyme biosensor systems. A glucose sensor will be fabricated using a kinetically doped silica sol−gel thin film through the coencapsulation of the glucose oxidase (GOD) and horseradish peroxidase enzymes. This is the first instance of more than one enzyme being included in the same sol−gel thin film and able to work together in a sequence of reactions with the product from one enzyme being used as the reactant for the other enzyme. This work demonstrates the advantages of the kinetic doping technique over existing modern GOD/HRP systems reported in the literature that despite using more laborious protocols, they usually result in lower concentrations of loaded enzyme found in ordered mesoporous silica,[19] macroporous silica foam,[20] and metal−oxide−semiconductor capacitors,[21] as well as allowing for a

Received: February 27, 2019
Revised: April 3, 2019
Published: April 4, 2019

DOI: 10.1021/acs.jpcb.9b01907
J. Phys. Chem. B 2019, 123, 3962−3967

visible colorimetric response compared to amperometric responses seen in other hybrid sol−gel membranes containing two enzymes.[22]

EXPERIMENTAL SECTION

Materials and General Methods. Tetraethylorthosilicate (TEOS), CytC (from equine heart), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, glucose, glucose oxidase (GOD), *o*-dianisidine, and Coomassie Brilliant Blue G-250 were purchased from Sigma-Aldrich. Phosphoric acid and hydrogen peroxide (30% solution) were purchased from EMD Millipore. HRP was purchased from Gold Biotechnology. Guaiacol was purchased from Cayman Chemical Company. Premium-grade glass coverslips (25 mm × 25 mm) were purchased from Fisher Scientific. Drain coating was performed via a variable flow chemical pump from Control Company. All chemicals and materials were used as received, with the exception of the glass coverslips, which were cleaned prior to use. All UV−vis spectra were obtained via a Shimadzu UV-2101PC UV/Vis Spectrometer.

Preparation of Glass Coverslips. To remove any organic contaminants on the glass coverslip surface, they were sonicated in an acetone bath for 30 min and rinsed with Millipore water three times to remove all acetone. The organic contaminant-free coverslips were then sonicated in 10% v/v NaOH for another 30 min and rinsed with Millipore water three times to remove all residual NaOH. The coverslips then went through a final sonication in Millipore water for 30 min to remove all traces of NaOH. Afterward, the coverslips were stored in Millipore water until use.

Preparation of Silica Sol. Silica sol was prepared by mixing 56.0 mL of TEOS, 111.7 mL of ethanol, 31.7 mL of Millipore water, and 0.620 mL of a 1% v/v phosphoric acid solution at room temperature, resulting in a 1:8:7 molar ratio of TEOS, ethanol, and water. The sol was allowed to age for 18 h at room temperature in the dark before use.

Preparation of GOD-, HRP-, and GOD/HRP-Doped Silica Sol−Gel Thin Films. Silica sol solution was transferred to a 400 mL beaker and elevated via a jack stand. A clean coverslip was purged-dried using compressed air and suspended from above so as to be immersed in the silica sol solution. The solution was drained from the beaker via a variable flow chemical pump such that the solution would recede from the sample coverslip at a rate of 80 mm/min. The flow was chosen according to previously reported methods[23] intended to result in a film of approximately 190 nm in thickness. Immediately following the removal of silica sol solution from around the newly coated coverslip, the jack stand holding the beaker was lowered until the coverslip was completely exposed. The newly made thin film was allowed to remain exposed to ambient air for another 5 min before it was transferred to an enzyme loading solution. GOD and HRP loading solutions consisted of 0.1 mg/mL GOD or HRP suspended in a 10 mM pH 7.4 phosphate-buffered saline (PBS) buffer. The dual-enzyme GOD/HRP loading solution consisted of 0.1 mg/mL GOD and 0.1 mg/mL HRP suspended in a 10 mM pH 7.4 PBS buffer.

Three different kinds of control samples for glucose detection were prepared: (i) control samples of enzyme loaded on bare glass were prepared by placing a cleaned glass coverslip directly into the dual GOD/HRP loading solutions; (ii) control samples of thin films with no enzyme loaded were prepared by soaking a nascent sol−gel film in a 10 mM pH 7.4 PBS buffer that contained no enzyme, and (iii) control samples of thin films loaded with only HRP.

Detection of Glucose Oxidase in Loaded Thin Films. After 1 week of kinetic doping in a GOD loading solution, thin-film samples were removed and washed under a direct stream of running distilled water to remove all GOD that are loosely bound to the thin-film surface. The presence of active GOD in the thin film was confirmed by a glucose/HRP/*o*-dianisidine solution assay. The assay solution was prepared by adding 1 mL of an 18% m/v (1 M) glucose stock solution and 0.5 mL of a 200 μg/mL HRP stock solution to 8 mL of 1% *o*-dianisidine in a 10 mM pH 7.4 PBS buffer stock solution. The assay was performed by directly submerging the GOD-loaded thin film in the assay solution. The presence of GOD in the silica film could be observed visually from the appearance of the colored oxidized *o*-dianisidine product, which slowly diffuses into the once colorless assay solution.

Detection of Horseradish Peroxidase in Loaded Thin Films. The HRP-loaded coverslips were removed after 1 week of kinetic doping in a HRP loading solution, which were then washed with distilled water to remove loosely bound enzymes. The presence of thin-film-loaded HRP was verified by a guaiacol solution assay. Guaiacol was chosen for the assay due to the dramatic color change from colorless to dark brown, as it is catalytically oxidized into the dimeric and tetrameric quinone products by HRP in the presence of H_2O_2.[13] The color change is easily observable even to the naked eye. The guaiacol assay solution is a mixture of 1.4 μL of 30% H_2O_2 and 3.3 μL of liquid guaiacol in 100 mL of a 10 mM pH 7.4 PBS buffer. The resultant H_2O_2 and guaiacol concentration in the assay solution are 140 and 300 μm, respectively. To prevent the inactivation of HRP by excess H_2O_2, very low H_2O_2 concentration was deliberately used in the design of this assay.[18] The assay was carried out by directly submerging the HRP-loaded thin film into the clear guaiacol assay solution. Subsequently, the presence of HRP could be visually confirmed by the formation of brown quinone products on the thin-film surface, which then gradually diffuses outward and spreads through the once colorless assay solution.

Quantification of HRP Catalytic Activity. The catalytic activity of thin-film-immobilized HRP was determined by following the rate of product formation in a HRP/guaiacol assay reaction.[24] The reaction was monitored in real time via the increase in the quinone product absorption at 436 nm under continuous stirring. The same experiment was repeated for both free HRP and HRP loaded in a thin film. The activity of immobilized HRP was calculated via the initial rate method by utilizing the initial linear portion of the 436 nm absorbance time course.

Quantification of GOD/HRP Tandem Catalytic Activity. The catalytic activity of thin-film-immobilized GOD/HRP was determined by following the rate of product formation in a GOD/HRP/guaiacol assay reaction. The guaiacol assay solution is a mixture of 1 mL of an 18% m/v (1 M) glucose stock solution and 3.3 μL of liquid guaiacol in 100 mL of a 10 mM pH 7.4 PBS buffer. The reaction was monitored in real time via the increase in the quinone product absorption at 436 nm under continuous stirring. The same experiment was repeated for both free HRP and HRP loaded in a thin film as a negative control. The combined activity of the GOD/HRP film was calculated from the initial linear portion of the 436 nm absorbance time course.

DOI: 10.1021/acs.jpcb.9b01907
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RESULTS AND DISCUSSION

Glucose Oxidase Detection. As this is the first instance of GOD being used in a kinetically doped thin film, it must first be shown to load into the thin film. To this purpose, a standard *o*-dianisidine assay was prepared and utilized to detect the presence of GOD loaded into the thin film. Figure 1 shows the

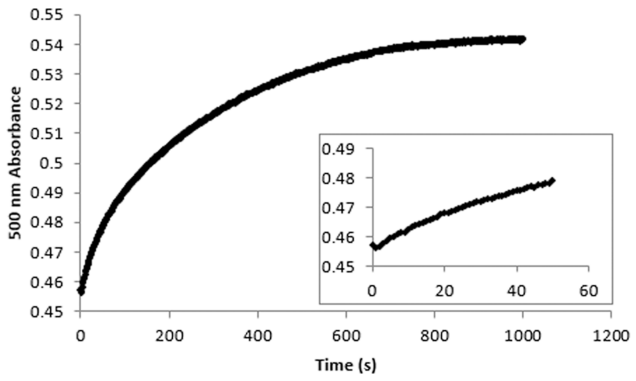

Figure 1. *o*-Dianisidine assay with glucose oxidase. Inset: initial linear portion.

resulting initial linear section of the reaction. As the reaction proceeds, it can be confirmed that GOD is present in the thin film, providing a reason to move forward with the multienzyme combination of GOD and HRP.

GOD Loading. The quantification of loaded GOD was carried out in a similar manner previously used for quantifying HRP-loaded thin films made via spin coating: the depletion of free Coomassie Brilliant Blue upon protein binding was monitored via the decrease in 465 nm absorbance over a 5 min period.[18] The experimental design used is arranged such that the sol–gel film, the loaded protein, and the glass coverslip are located away from the optical path of the UV–vis spectrometer to prevent the Coomassie-Brilliant-Blue-stained thin film from blocking the optical path and interfering with the measurement. The calibration curve in Figure 2 shows the decrease in

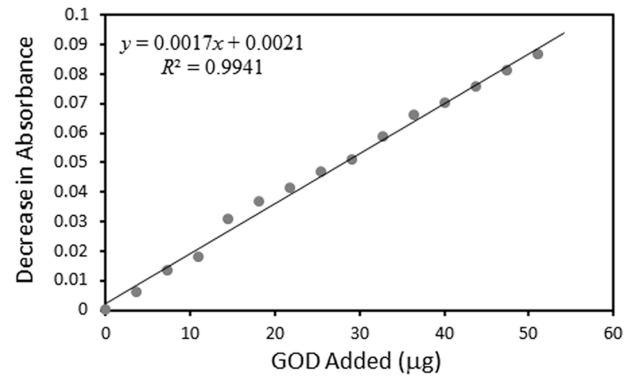

Figure 2. Calibration curves for glucose oxidase generated via an adapted Bradford assay against known enzyme concentrations.

the 465 nm absorbance of Coomassie Brilliant Blue upon the addition of known amounts of GOD and indicates a reasonably long linear range from which the mass equivalent of thin-film-loaded proteins can be estimated.

After performing the Bradford assay on the GOD-loaded thin films, the average change in absorbance, determined from four replicate runs, was 0.016 ± 0.001. From the calibration curve, this recorded change in absorbance corresponds to 0.009 ± 0.001 mg of solution-accessible GOD in a single thin-film-coated coverslip. This shows that the thin film had removed 0.92% of the GOD present in the enzyme loading solution. When taking into account the previously known thickness of the thin film of 190 ± 10 nm, the coverslip surface area of 25 × 25 mm^2,[23] which is then doubled to account for dip coating on both sides of the glass coverslip, and an uncoated corner area of 12.25 mm^2 on both sides of the glass coverslip that is always held above the sol solution during drain coating, the volume of the thin film can be calculated. The resulting volume can then be used to determine the concentration of solution-accessible GOD in the thin film as 0.25 mmol/L. This represents approximately a 400× increase in concentration compared to the GOD loading solutions of 0.0008 mmol/L. It is worth noting that these numbers only take into consideration the solution-accessible GOD, which is the amount of dye-accessible GOD, as any protein inaccessible to the Coomassie Blue dye in the short 5 min time frame of the Bradford assay would not be revealed by the assay. With the relatively large size of Coomassie Blue ($C_{47}H_{48}N_3NaO_7S_2$, FW: 854.02 g/mol), it is unlikely to diffuse through the porous thin-film structure efficiently, meaning it is quite possible that the actual amount of GOD in the thin film is most likely greater than that reported by the Bradford assay.

Table 1 compares the loading of GOD to known loading values of HRP in our previous study.[23] It is interesting to note that GOD loads significantly less than HRP, which is probably due to the difference in size between the two enzymes. The HRP enzyme is approximately 44 kDa, while GOD consists of two subunits, each 80 kDa, for a total of 160 kDa. With the GOD being so considerably larger, it stands to reason that fewer loading sites in the film would be sufficiently large to accommodate GOD during the kinetic doping process. A potential implication is that once immobilized, the larger GOD may block off many sites that are suitable for trapping the smaller HRP.

Kinetically doped thin films loaded with HRP and GOD together were also subjected to the Bradford assay to measure the total enzyme loading. Here, the average decrease in absorbance at 465 nm was found to be 0.032 ± 0.006. This value can be used in conjunction with the overall activity value of the thin film to estimate the mass of each protein present. Assuming that the thin films kinetically doped with only one enzyme represent "fully loaded" thin films and that the GOD/HRP-loaded thin film has the same number of available loading sites that HRP and GOD must compete for results in the GOD and HRP loading amounts being a fraction of what they would be in a fully loaded thin film of the respective enzyme.

GOD/HRP Activity. To serve as a multienzyme biosensor, it is not enough for the enzymes simply to be present; they must be able to work together with the substrate that the sensor is meant to detect. As such, the substrate must be able to reach and react with the first enzyme, and the product of the first reaction must be able to reach the second enzyme to produce the final observed product. In the case of the GOD/HRP-loaded thin film, the glucose being tested for must be able to reach the immobilized GOD and produce H_2O_2. The freshly produced H_2O_2 must, in turn, be able to reach the HRP and catalyze the transformation of guaiacol to the colored quinone dimer. This is characterized by the overall activity of the GOD/HRP-loaded thin film. The overall activity was measured via a stock assay solution that contains 1 mL of 18%

DOI: 10.1021/acs.jpcb.9b01907
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Table 1. Enzyme Loading in Silica Sol–Gel Thin Films

sample	average ΔA	average enzyme loading (mg)	conc. of soaking solution (mmol/L)	percent of soaking dopant loaded (%)	enzyme concentration in thin film (mmol/L)	increase over soaking solution
horseradish peroxidase	0.052 ± 0.009	0.055 ± 0.009	0.0023	5.5	5.4 ± 0.5	2400×
GOD	0.016 ± 0.001	0.009 ± 0.001	0.0008	0.92	0.25 ± 0.01	320×
HRP/GOD	0.032 ± 0.006					
bare coverglass	0.001 ± 0.002	0.001 ± 0.002	0.008	0.08		

m/v glucose and 3.3 μm guaiacol in 99 mL of a 10 mM pH 7.4 PBS buffer, where a distinctively brown quinone product forms on the thin-film surface before diffusing to the rest of the solution. The quantity of the brown quinone dimer produced can be monitored via UV–vis spectroscopy, as shown in Figure 3. A control sample of a thin film kinetically doped with only HRP is also included (dashed line) to show that the production of quinone dimer only commences when both GOD and HRP are present. The overall activity can be determined by examining the initial linear portion of the 436 nm absorbance trace shown in Figure 4.

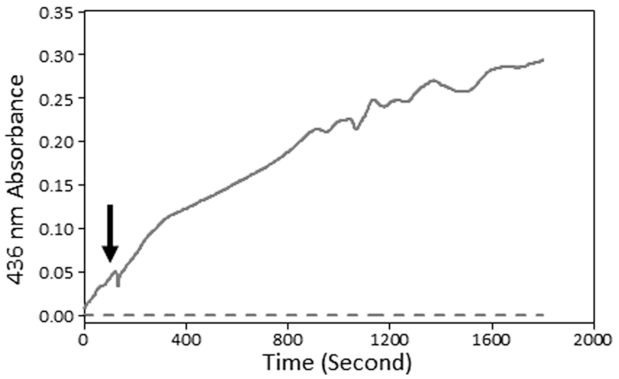

Figure 3. Activity of GOD/HRP-loaded (solid line) and HRP-only-loaded (dashed line) thin films. The arrow denotes the cutoff location for Figure 4.

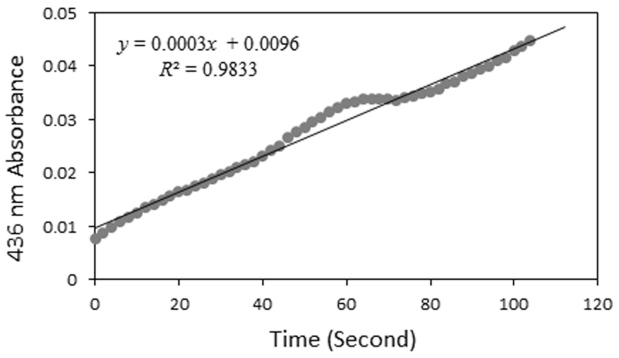

Figure 4. Initial linear portion of GOD/HRP activity assay.

The initial activity can be calculated from the rate of guaiacol consumption via eq 1

$$\text{activity} = \frac{\Delta A_{436\text{nm}} \times 2 \times V_t}{t \times \varepsilon} \quad (1)$$

where V_t is the total assay solution volume, ε = 25.5 mM^{-1} cm^{-1} is the extinction coefficient of the brown quinone dimer at 436 nm,[25] t is the time in minutes, and 2 is a constant related to the stoichiometric ratio of guaiacol to quinone dimer product per reaction.

A major assumption in this calculation is that the overall rate is limited by the HRP-catalyzed oxidation of guaiacol due to a much higher GOD activity than HRP in free solution, which is further compounded by the easier ingress and egress of the H_2O_2 produced by the GOD compared to the significantly bulkier quinone product produced by the HRP. This assumption is used, along with the activity resulting from GOD/HRP thin film relative to the activity resulting from a thin film fully loaded with only HRP, to give an estimate of the amount of HRP loaded in a GOD/HRP thin film. The overall activity (U) from the GOD/HRP thin film gives 0.038 U, while a thin film fully loaded with only HRP results in an activity of 0.114 U.[19] Since the activity of the GOD/HRP thin film is 33.3% the activity of the fully loaded HRP thin film, the HRP content in a GOD/HRP thin film can be estimated as 33.3% that of the HRP-only thin film, indicating that it contains 0.018 ± 0.003 mg HRP.

Under the assumption that the absorbance result from the Bradford assay of the GOD/HRP thin film given in Table 1 is a linear combination of the contributions from GOD and HRP, the estimated HRP mass of 0.018 ± 0.003 can be used to estimate the mass of GOD from the Bradford assay results of the fully loaded thin films and the GOD/HRP-loaded film following eq 2

$$(A_{\text{F-GOD}} \times f_{\text{GOD}}) + (A_{\text{F-HRP}} \times f_{\text{HRP}}) = A_{\text{Obs}} \quad (2)$$

where $A_{\text{F-GOD}}$ is the resulting absorbance from a Bradford assay of a thin film fully loaded with GOD, f_{GOD} is the fraction of the mass of GOD in the dual-enzyme film relative to a fully loaded GOD thin film, $A_{\text{F-HRP}}$ is the resulting absorbance from a Bradford assay of a thin film fully loaded with HRP, f_{HRP} is the fraction of the mass of HRP in the dual-enzyme thin film compared to a fully loaded HRP thin film loaded, and A_{Obs} is the resulting absorbance measured from a GOD/HRP thin film. Using f_{HRP} = 0.333 determined for HRP based on the activity of the dual-enzyme thin film relative to that of a fully loaded HRP film, f_{GOD} can be determined to be 0.917, corresponding to 0.008 ± 0.001 mg of GOD loaded into the GOD/HRP thin film. Table 2 below summarizes this data for the GOD/HRP thin film.

The assumption about the HRP reaction being the rate-limiting step can be verified by a comparison of the reaction rate measured from calculated concentrations of thin-film-immobilized enzymes to similar concentrations of free enzyme

Table 2. Individual Enzyme Loading in Dual-Enzyme Silica Sol–Gel Thin Films

enzyme	mass loaded (mg)	concentration in thin film (mmol/L)
HRP	0.018 ± 0.003	1.8 ± 0.1
GOD	0.008 ± 0.001	0.22 ± 0.01

DOI: 10.1021/acs.jpcb.9b01907
J. Phys. Chem. B 2019, 123, 3962–3967

in solution. This can be seen in Figure 5, which shows the initial linear portion of the reaction rate from 54 mmol/L of free HRP in solution, approximately 10 times the concentration of HRP found in an HRP-only thin films,[22] and 188 mmol/L of free HRP and 2.2 mmol/L of free GOD in solution, which is approximately 10 times the amount predicted to be trapped in the tandem thin film shown in Table 2. In order for the assumption to be valid, the activity must show a similar ∼66% decrease in the GOD/HRP reaction rate relative to that of the HRP-only reaction. That this roughly is the case can be seen in the decrease in the rate of change in 436 nm absorbance in the GOD/HRP reaction at 27% relative to the HRP-only reaction. The slight additional decrease in activity (27% in solution vs 33% in GOD/HRP film) may be caused by the larger amount of H_2O_2-accessible HRP relative to the Coomassie Brilliant Blue dye-accessible HRP measured from the assay adapted from Bradford.

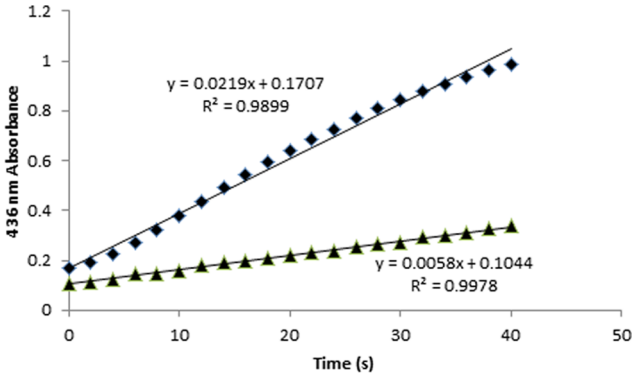

Figure 5. Initial linear portion of free HRP (diamond) and free HRP/GOD (triangle).

The data shows that the activity of the dual-enzyme system thin film loses approximately two-thirds of its activity compared to that of a single-enzyme-loaded film, not an unexpected loss due to having multiple enzymes competing for limited loading spaces inside a thin film. With a bulkier dimension and heavier mass than the HRP, GOD is shown to be more efficient at competing for these loading sites. This is substantiated by the retention of 91.7% GOD loading as opposed to that of 33.3% HRP loading in the dual-enzyme thin film. These results demonstrate that the kinetically doped dual-enzyme thin film retains the characteristic loading efficiency, achieving concentrations as high as 1.8 mmol/L from just a 0.0023 mmol/L loading solution, granting an advantage over other forms of silica used with GOD/HRP systems reported in the literature, requiring less processing intensive manufacturing, while maintaining comparable if not somewhat advantageous activity rates relative to other most recent GOD/HRP systems.[19−22]

Further fine-tuning of the amount and ratio of each enzyme loaded may be possible by controlling the concentration of each enzyme in the loading solution. The method for calculating the mass loaded will still be applicable provided the information about the full loading capacity of a thin film for every single enzyme at each concentration be determined first. This can potentially allow for additional fine-tuning of performance to achieve the most optimized reaction rate. Similar to our previous study on thin films loaded with only one enzyme, the dual-enzyme thin films also show a noticeable reduction in maximum response after repeated use.[23] Methods of refreshing the thin film that could alleviate this and restore activity present opportunities for further research.

CONCLUSIONS

In this study, the dual-enzyme, multistep biosensor produced through kinetic doping was constructed to detect the presence of glucose successfully. These dip-coated thin films, simultaneously loaded with horseradish peroxidase and glucose oxidase, proved to be capable biosensors. Most importantly, it was easily achievable through the kinetic doping technique, allowing for ease of creation beyond many current sol−gel encapsulation techniques without sacrificing an enzyme friendly environment. The success of this very first dual-enzyme kinetically doped biosensor demonstrates that the kinetic doping process is easily capable of loading sufficient amounts of enzymes even though the limited amount of loading sites in a thin film is split between multiple enzymes. The resulting activity in this dual-enzyme film is still sufficiently high to produce a viable biosensor, opening the door to a much wider variety of more complex biosensors utilizing more than two enzymes in the future.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpcb.9b01907.

HRP/GOD assaying in guaiacol assay (AVI)

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Author Contributions

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

This work was supported by the Oklahoma Centre for the Advancement of Science and Technology (HR 12-128).

Notes

The authors declare no competing financial interest.

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DOI: 10.1021/acs.jpcb.9b01907
J. Phys. Chem. B 2019, 123, 3962−3967