Published in final edited form as:

*IEEE Trans Nanobioscience*. 2003 September ; 2(3): 133–137.

# Multilayer Lactate Oxidase Shells on Colloidal Carriers as Engines for Nanosensors

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## Abstract

Self-assembly methods are being studied for construction of nanoscale chemical sensors employing coimmobilized enzymes and indicator dyes. This paper describes the assembly of the catalytic enzyme lactate oxidase in multilayer films on colloidal templates via layer-by-layer self-assembly, which is a step toward achieving nanoengineered biosensors. The architecture of the resulting films was characterized using quartz crystal microbalance and zeta potential analysis, and catalytic activity was characterized colorimetrically. The tailored activity of the functional nanofilms was proportional to the number of enzyme layers deposited during assembly, which provides a basis for designing sensors with specific interactions.

### Index Terms

Electrostatic layer-by-layer assembly; lactate oxidase; multilayer thin films; nanoreactor

## I. Background

Optical sensors for nanoscale analysis are desirable for biological studies and potential clinical application. Such systems for monitoring biochemical substrates require a combination of specific molecular recognition and signal transduction that is difficult to achieve. Enzyme-based biosensors are attractive because of the inherent specificity of the enzyme–substrate interaction. Cosubstrates such as oxygen or reaction products, such as a change in local pH or $O_2$ levels, have been demonstrated as means to quantify substrate concentrations. To approach the goal of nanoscale sensors, it is necessary to develop a means of colocalizing the enzyme and indicator. This colocalizing procedure should provide control over the relative amounts of enzyme and indicator per sensing unit in a mild environment.

Traditional enzyme immobilization techniques include Langmuir–Blodgett, antigen–antibody interactions, self-assembled monolayers, surfactant films, and avidin–biotin interactions [1]. In this paper, the immobilization technique chosen is electrostatic layer-by-



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layer assembly (LbL) adsorption [2], [3], [4, p. 125], [5], a technique first presented by Iller in 1966 [6], which illustrated the adsorption of charged colloids to a solid substrate. After Decher demonstrated the preparation of ultrathin films using the LbL technique [7], this technique has received considerable interest recently, due to the ability to precisely control film thickness and composition [8]. The LbL technique has been successfully applied to the assembly of linear polyions, proteins, viruses, ceramics, lipid tubules, and charged nanoparticles [4, p. 127].

The LbL protocol involves placing a charged solid template—in this paper, polystyrene (PS) particles—in a solution of oppositely charged species, at a pH providing a high degree of ionization, for a given amount of time. The charged material adsorbs to the charged surface due to electrostatic and other attractive forces in an approximate monolayer configuration, resulting in surface charge reversal. The unadsorbed polyionic species is then removed, usually through a series of centrifugation cycles, followed by the introduction of an oppositely charged species. This process is then repeated until the desired thin-film architecture is realized on the template surface.

Previous studies have shown the LbL assembly of enzymes for the purpose of biocatalysis [8], [9], [10]. These nanoreactors offer increased surface area in which the catalysis takes place, the ability to recover the enzyme, and the capability to dope polyions with molecules such as dyes, which can be used to monitor reaction activity [4, p. 163]. The ability to tailor order and complexity during the fabrication of nanoreactors through LbL assembly offers advantages over the copolymer or liposome reactors traditionally used [11], [12].

In this paper, the precise assembly of lactate oxidase (LOx) multilayers on 520-nm PS particles, with engineered enzymatic activity, is reported. The layering properties and the enzymatic activity of LOx embedded in polyelectrolyte films were quantified. These fundamental properties of LOx will play a crucial role in the design and fabrication of nanoscale fluorescent sensors for lactate, which have potential clinical applications such a minimally invasive monitoring of brain and blood lactate concentrations. LOx catalyzes the oxidation of l-Lactate into pyruvic acid, as seen is the following reaction: l-lactic Acid + $O_2$ $\rightarrow$ Pyruvic acid + $H_2O_2$ [13], [14]. Accordingly, monitoring local oxygen, pH, or peroxide with LOx catalysis results in a specific biochemical sensor. Thus, coimmobilization of indicator dyes with the enzymes can be used to construct such nanodevices.

## II. Experimental

LOx (Pediococcus species, Sigma, St. Louis, MO) was used at a concentration of 1.5 mg/mL in deionized water. The Mw of LOx is ~80 kDa, and the isoelectric point is ~pH 4.6 [15], such that the enzyme has net negative surface potential during assembly under neutral pH conditions. Poly(dimethyldiallylammoniumchloride) (PDDA; Aldrich, Milwaukee, WI, Mw 100–200 kD) and Poly(styrenesulfonate) (PSS; Aldrich, Milwaukee, WI, Mw 70 kD) solutions were prepared in deionized water to a concentration of 2 mg/mL. PS spheres with an average diameter of 520 nm were obtained from Seradyn, Ramsey, MN. The LOx activity assay consisted of *o*-Dianisidine dihydrochloride (Sigma, St. Louis, MO),

Peroxidase (Type II: from horseradish, Sigma, St. Louis, MO), and l-Lactic Acid (Fluka, Buchs, Switzerland). All experimental procedures were performed at room temperature.

Alternate polyions/LOx adsorption cycles were monitored using quartz crystal microbalance (QCM) analysis with resonators having evaporated silver electrodes with combined surface area of 0.16 $cm^2$ and initial resonance frequency of 9 MHz (AT-cut). During the assembly procedure, each resonator was immersed in a polyion solution for 15 min to allow complete surface saturation, rinsed by immersion in water, and dried with streaming nitrogen; then, resonance frequency was measured [3], [5]. For enzyme deposition, the resonator was immersed in the LOx stock solution for 20 min to allow adequate time for complete surface coverage to take place. Two precursor bilayers of {PDDA/PSS} were implemented to ensure that the solid substrate exhibited a smooth and uniformly charged foundation prior to the addition of the enzyme multilayers [5]. The change in resonance frequency of the QCM was monitored for each adsorption step.

For assembly of films on microtemplates, approximately $10^{11}$ 520-nm PS particles were added to a 2-mL centrifuge tube along with the subsequent addition of polyions and LOx to yield the following structural design: $\{(PDDA/PSS)_2/PDDA/(LOx/PDDA)_{1–3}\}$, where subscript 1–3 indicates that 1, 2, and 3 bilayers of LOx/PDDA were deposited in separate assembly experiments. Following the addition of the polyions on to the solution of PS particles, the latex–polyion mixture was allowed to rest for 15 min to allow complete saturation of the particle surface by the polyions. After the addition of $\{(PDDA/PSS)_2/$ PDDA} to the surface of the particles, the particle dispersion was then separated into three equal portions, with each receiving $\{LOx/PDDA\}_{1–3}$, respectively. The PS particles were dispersed in the enzyme solution for 45 min. A greater length of time was allotted for the enzyme deposition step on the PS particles when compared to the QCM resonator to ensure complete coverage of the large surface area of the particles. The additional adsorption time of the enzyme deposition on the 520-nm PS particles does not affect the average thickness of the deposited film layer, because once monolayer coverage of negatively charged enzyme is reached, further adsorption is opposed by electrostatic repulsion [19]. In between each adsorption step, the latex/polyion/LOx solution was centrifuged at 18 000 g for 13 min, followed by the removal of the supernatant, which contained the unadsorbed enzyme. This procedure was repeated four times per adsorption step to eliminate admixing of oppositely charged species [9], [10]. The amount of enzyme present at each step was quantified through UV-Vis spectrophotometry by comparing the enzyme concentration after adsorption to enzyme concentration prior to adsorption. A Brookhaven Instruments ZetaPlus zeta potential analyzer and an Agilent 8453 UV-Visible Spectrophotometer were used to characterize the growth of the enzyme multilayers on PS particles.

LOx activity was monitored through a colorimetric assay based on the oxidation of *o*-Dianisidine through a peroxidase coupled system: l-lactic Acid + $O_{2-} \rightarrow$ Pyruvic acid + $H_2O_2$ + Reduced *o*-Dianisidine (noncolored) $\rightarrow H_2O$ + Oxidized *o*-Dianisidine (colored). The enzymes LOx and peroxidase catalyze the series of reactions. The reaction results in an increase in absorbance at 500 nm as a function of LOx concentration. For the assay, a 0.05-M sodium acetate buffer was adjusted to pH 5.1 through the addition of 1 M HCl, followed by the addition of *o*-Dianisidine to a concentration of 0.21 mM. A peroxidase solution, with

a concentration of 60 Purpurogallin units/mL, and a solution of 0.1 mg/mL of l-Lactic acid were prepared in deionized water. The LOx activity assay comprised 2.4 mL of *o*-Dianisidine solution, 0.5 ml of l-Lactic acid solution, and 0.1 mL of peroxidase solution. During continuous stirring with a magnetic bar, a known amount of LOx multilayer particles was added to the assay, and the absorbance at 500 nm was monitored as a function of time, resulting in a catalytic profile of the multilayered nanoreactors.

## III. Results and Discussion

In order to examine the layering properties of LOx, enzyme multilayers were first constructed on QCM electrodes. The Sauerbrey equation relates the frequency shift caused by the adsorbed mass on the resonator [16]. The frequency shift (ΔF, Hz) of the resonator is related to mass deposited (ΔM, ng) [5] by:

$$\Delta M = -0.87\Delta F \quad (1)$$

which results in a change in thickness (ΔL, nm) of the film [17]

$$\Delta L = -0.017\Delta F \quad (2)$$

The measured frequency shift of the deposited species is reflected in Fig. 1, which presents the changes in thickness resulting from deposition of the following multilayer architecture: $\{(PDDA/PSS)_2/PDDA/(LOx/PDDA)_{1–3}\}$.

The regular stepwise decrease in frequency for LOx addition corresponds to an average thickness of 8.5 nm and an average mass coverage of 13.6 mg/m$^2$ per layer of LOx.

The techniques used for successful assembly of LOx multi-layers of QCM substrates were then applied to form LOx multi-layers on 520-nm PS spheres. The precursor film consisting of $\{(PDDA/PSS)_2\}$ was also applied to the colloidal template to provide even surface coverage and charge distribution [5]. The addition of LOx multilayers to the PS particles was monitored through microelectrophoresis. Fig. 2 depicts the zeta potential of the latex particles' surface due to deposition of polyions and LOx $\{(PDDA/PSS)_2/PDDA/(LOx/PDDA)_{1–3}\}$.

The alternation of positive and negative zeta potential values corresponds to the adsorption of the positive and negative species, respectively. These values also quantify the complete change in surface potential of the PS particles, which is critical in LbL assembly of multilayer films [18]. The zeta potential measurements taken after the adsorption of a LOx layer exhibit a lesser negative potential value than that of the PSS layers, which is expected because the isoelectric point of LOx, ~pH 4.6, is closer in value to the neutral assembly conditions than the pI of PSS (~pH 1).

The extraction of LOx from stock solutions during subsequent layer addition was examined through UV/Vis spectrophotometry. Measurement of the initial LOx stock solution (1.5 mg/mL) prior to layer adsorption was compared to the LOx concentration in the supernatant after layer adsorption. The absorbance spectrum for the initial LOx solution, with a

concentration of 1.5 mg/mL, is shown in Fig. 3, along with spectra collected for the stock solution following a single adsorption step.

The absorbance spectrum of the initial solution of LOx after adsorption and centrifugation was used to quantify the decrease in LOx concentration attributed to thin-film formation. The change in absorbance corresponds to a decrease in LOx concentration of 21%, indicating that approximately 21% of the LOx present in the initial solution was adsorbed onto the PS particles. This translates into an adsorption of 0.332 mg of LOx onto the PS particles, which agrees well with the value of 0.291 mg of LOx estimated using the available surface area of the particles ($cm^2$) and applying the average mass coverage ($mg/cm^2$) calculated from the QCM results.

The supernatant following PDDA adsorption was also measured, which is given by spectrum 3 in Fig. 3, and examined to determine whether significant LOx was desorbed from the surface during the subsequent adsorption of PDDA. This behavior has been observed in other studies for Urease [9]. On the basis of a lack of an observable absorbance peak at 280 nm, it was concluded that there was no evidence of LOx extraction during the subsequent addition of PDDA.

Following successful deposition of LOx multilayers, the enzyme activity of the particles with multilayer film coatings was examined. Fig. 4 shows the catalytic activity of the LOx multilayered PS particles, which suggests an approximately linear increase in enzymatic activity as a function of adsorbed LOx layers. The catalytic activity reflects that of the $\{(PDDA/PSS)_2/PDDA/(LOx/PDDA)_{1–3}\}$ particles, and data are normalized according to the estimated number of particles in the respective solutions. By normalizing the data to the number of particles in the respective solutions, the relative effect of particle concentration of the respective solutions is removed, such that the deposited mass of LOx is accountable for the observed enzymatic activity. The number of particles present was calculated using a model which estimated ~8% loss of particles during quadruple centrifugation [8].

The catalytic activity of the enzyme films was analyzed through examination of the slopes of the absorbance profiles after 100 s of reaction. The activity of one, two, and three multilayered LOx PS particles after 100 s of elapsed activity was calculated to be $2.42 \times 10^{-3}$, $4.66 \times 10^{-3}$, and $7.09 \times 10^{-3}$ absorbance units/sec, respectively, as indicated in Fig. 5. The increase in activity shows approximately linear relationship with increasing layers of enzyme. Upon normalization of the activity data presented in Fig. 5 to the estimated mass of LOx present on the respective particles, it was found that the activity of the respective solutions varied approximately 10%. This value is expected, considering that LOx is the contributor to the catalytic activity of the nanoreactors, and by normalizing the data to the estimated mass of LOx present, the effects of LOx mass are compensated; therefore, the activities of the respective particle dispersions are similar.

In order to compare the enzymatic activity of the multilayer lactate nanoreactors to that of free LOx, the enzymatic activity of free LOx must be experimentally determined. Using the colorimetric activity assay previously described, the activity of free LOx was to be $2.246 \times 10^{-3}$ absorbance units/s. Particles with one, two, and three enzyme layers exhibited 10.7%,

20.7%, and 31.5% of the activity of free LOx in solution. Thus, the enzyme films show decreased efficiency of catalysis compared to free enzyme. The differences in the catalytic activity of free LOx to that of entrapped LOx on the surface of multilayered particles can be attributed to substrate diffusion limitations and difficulties in reaching the active sites of the immobilized LOx, as has been suggested in studies with other enzymes immobilized in LbL films [8]. Despite this decreased efficiency, the ability to deposit dense layers of enzymes in ultrathin films with precise control and retained activity gives rise to the potential for nano-engineering highly specific biosensors.

## IV. Conclusion

Polymer-enzyme multilayered ultrathin films containing LOx have been prepared and characterized accordingly. Mass deposition measurements on QCM resonators showed stable layering properties of LOx, and zeta potential measurements confirmed the alternation of surface potential on 520-nm PS particles. Calculations of deposition density showed LOx is immobilized on charged surfaces with high efficiency in alternation with polyions. The catalytic activity of these colloidal nanoreactors was shown to increase linearly with the addition of one, two, and three enzyme multilayers. These nanoreactors have the benefits of large surface area and recoverable enzyme substrates through isolation to the particle surface, making them attractive for use in nanosensor applications.

## Acknowledgment

The authors would like to thank Dr. Y. Lvov for allowing use of the QCM and zeta potential analyzer.

This work was supported in part by the Whitaker Foundation Grant RG-00-0388 and in part by National Science Foundation Grant 0092001.

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## Biographies

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Dr. McShane is a member of the IEEE Engineering in Medicine and Biology Society, the Biomedical Engineering Society, the American Institute of Chemical Engineers, SPIE, the Society for Applied Spectroscopy, and Tau Beta Pi.

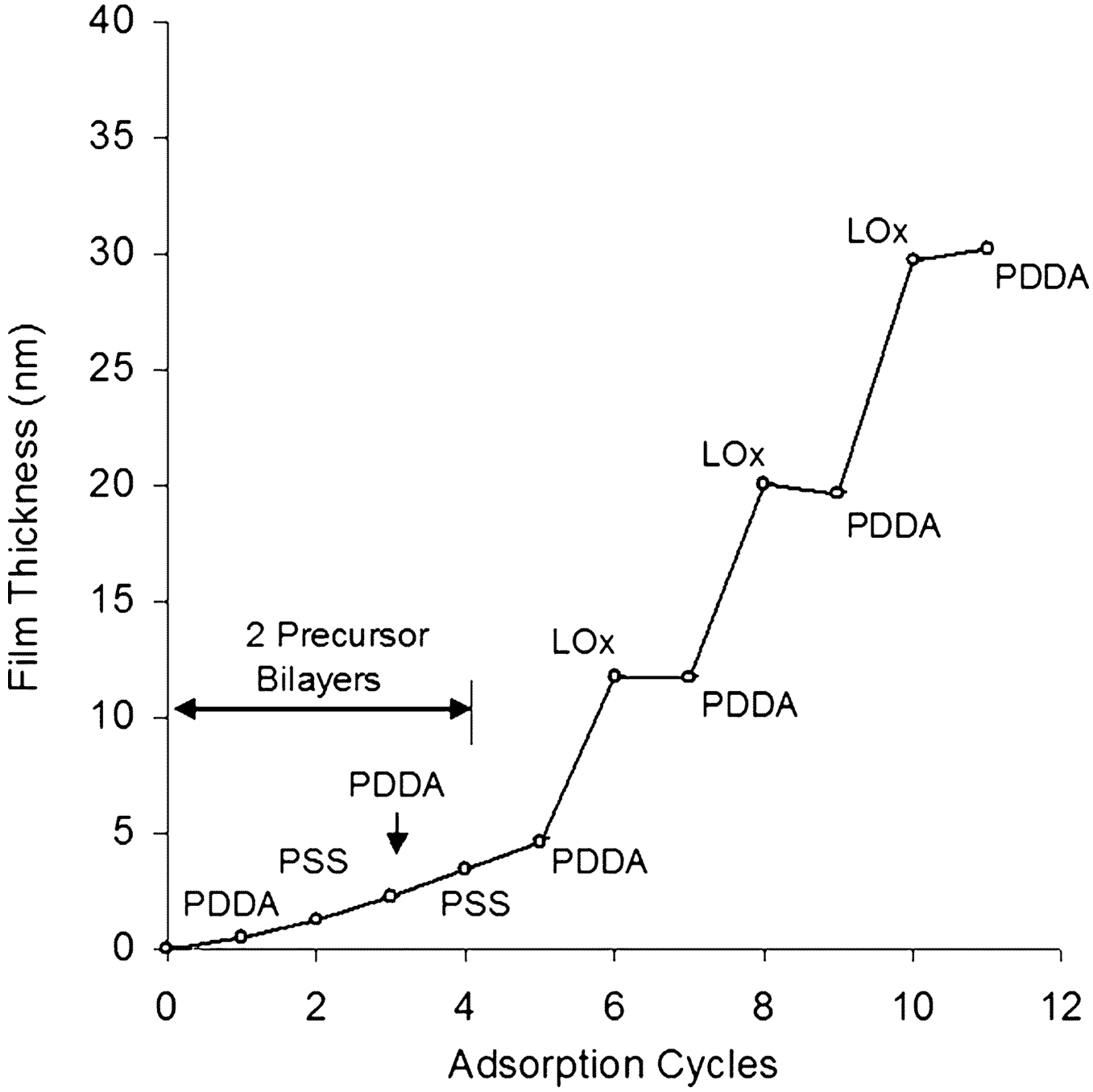


**Fig. 1.**

Film thickness for adsorption of PDDA alternated with PSS and LOx. Linear growth is apparent in the precursor bilayers, followed by stepwise increase in film thickness through the adsorption of LOx.

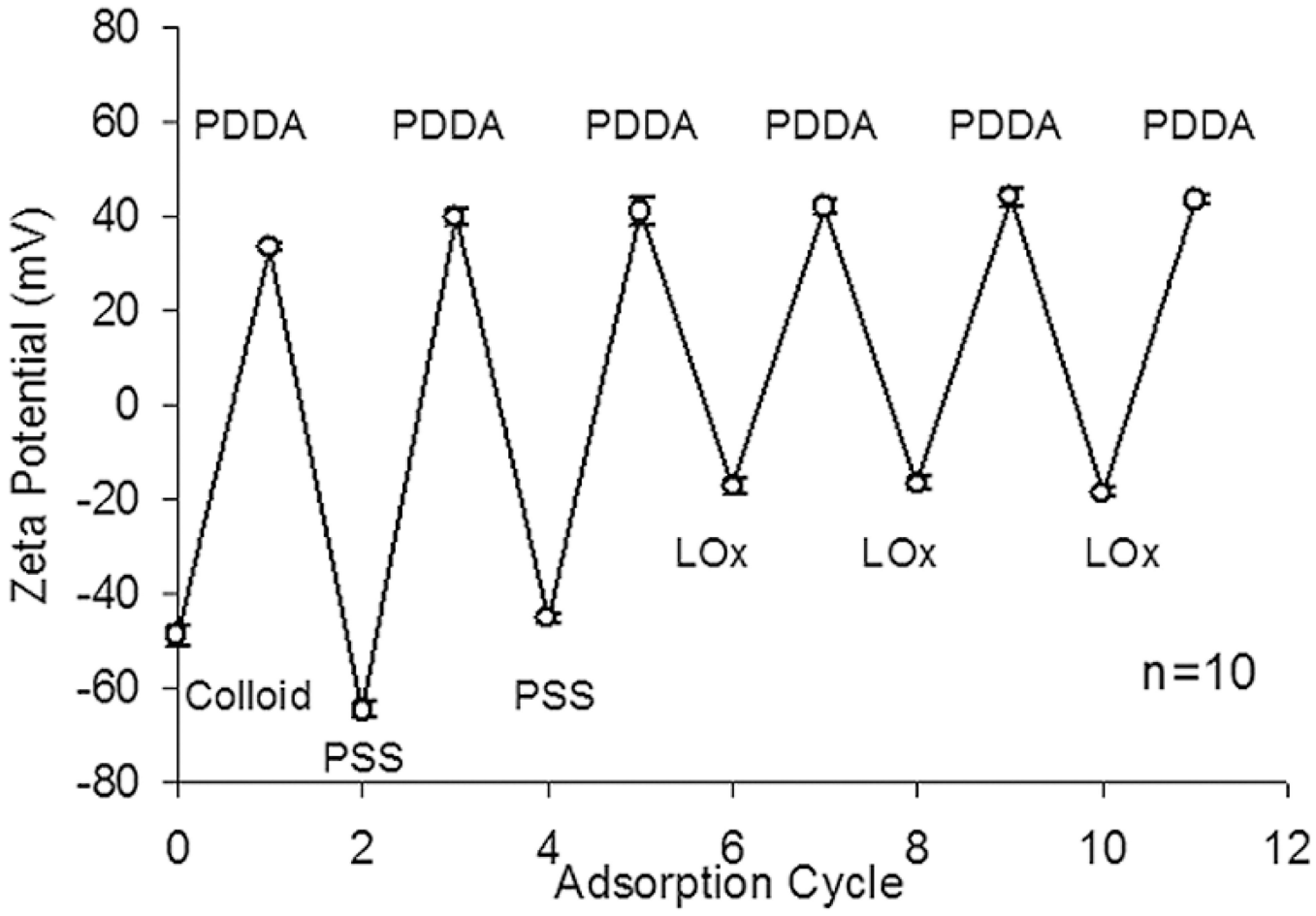


**Fig. 2.**

Zeta potential of {(PDDA/PSS)$_2$/PDDA/(LOx/PDDA)$_{1–3}$} multilayers on 520-nm PS particles.

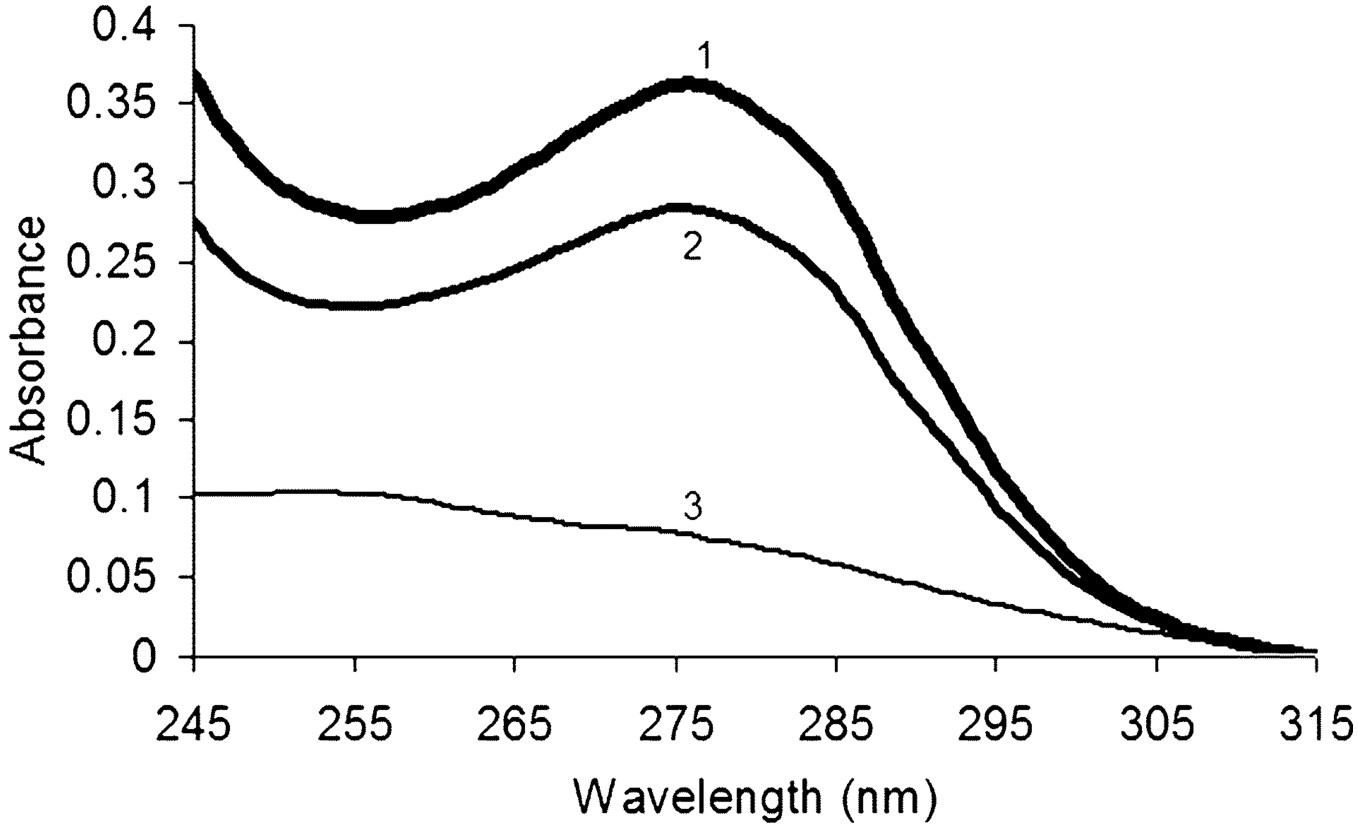


**Fig. 3.**
UV/Vis spectra of (1) LOx solution prior to adsorption, (2) LOx solution after adsorption, and (3) PDDA solution after adsorption.

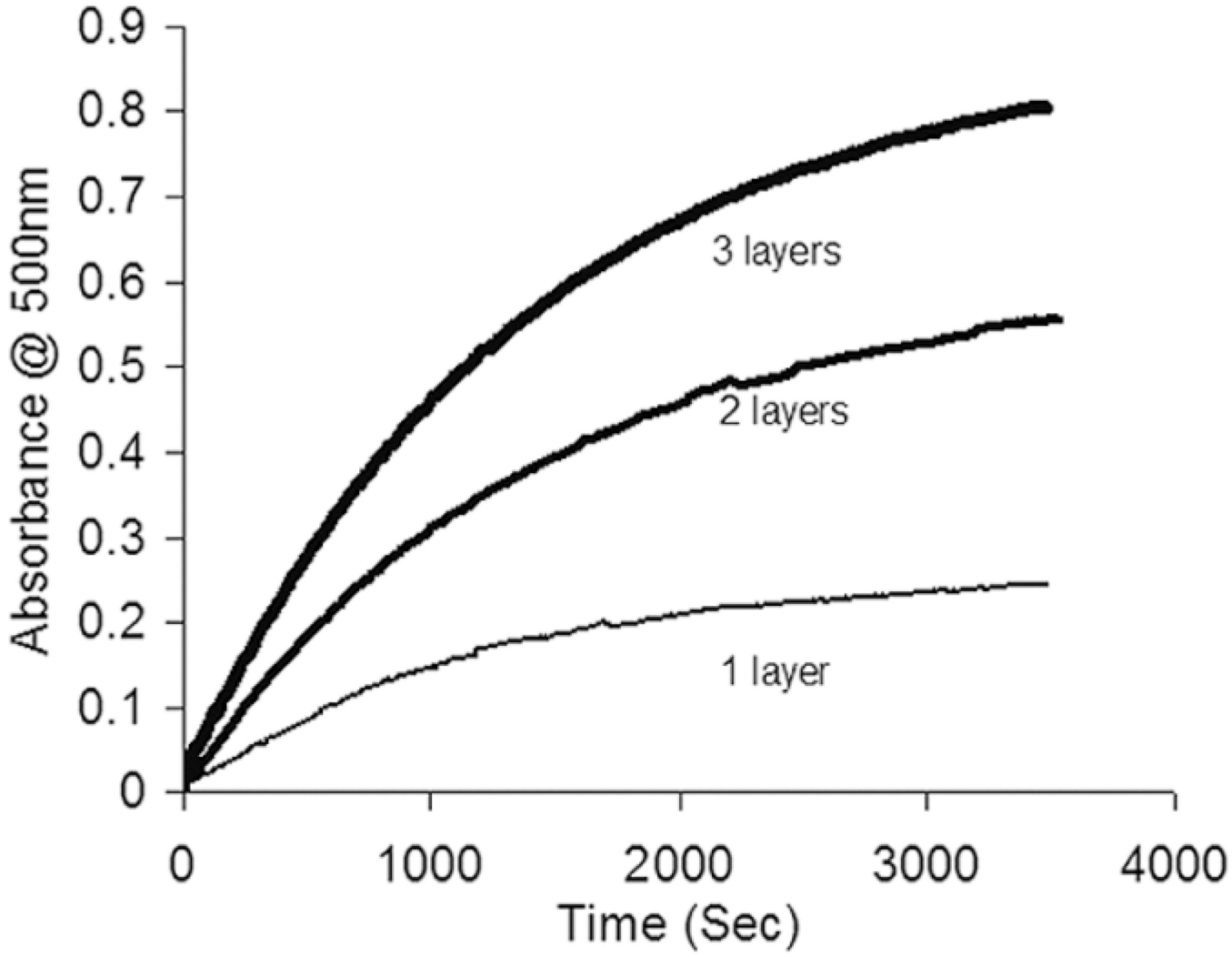


**Fig. 4.**

Enzymatic activity of LOx nanoreactors containing one, two, and three layers of LOx, respectively.

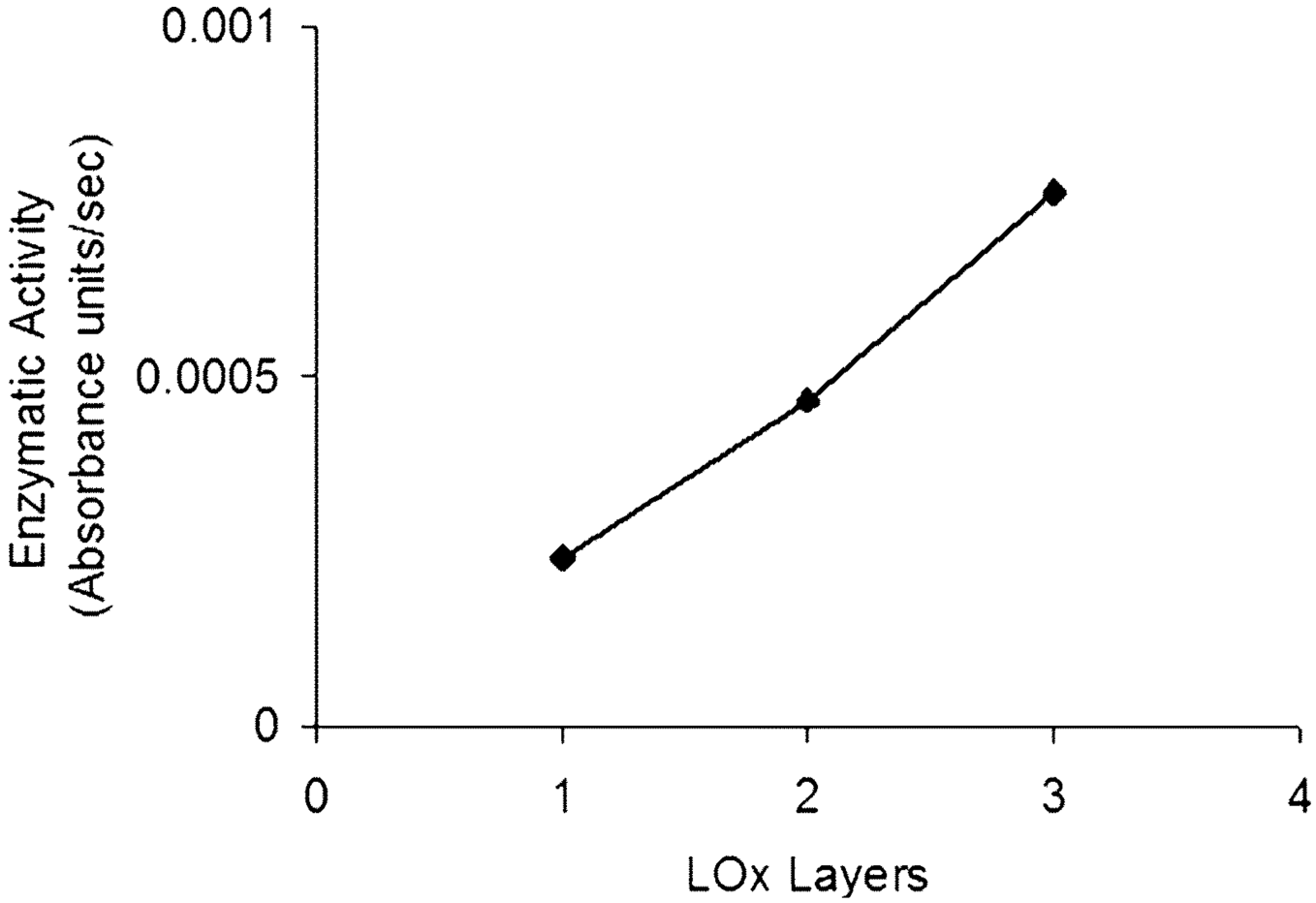


**Fig. 5.**
Enzymatic activity after 100 s of nanoreactors containing one, two, and three layers of LOx, respectively.