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# Glucose Oxidase Multilayer Modified Microcantilevers for Glucose Measurement

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**We report a novel enzyme-based microcantilever sensor by using layer-by-layer nanoassembly modification. A glucose oxidase (GOx) functionalized microcantilever underwent bending when it was exposed to glucose solutions. The magnitudes of bending were proportional to the concentrations of glucose. The cantilever bending was specific toward glucose, but not to other sugars such as mannose, fructose, or galactose. The flow rate effect on the cantilever bending response is also discussed. The bending mechanism was investigated, and the kinetic and thermodynamic analysis and experimental results showed that the conformational change of GOx and gluconic formation were the origin of cantilever deflection.**

Accurate and in-time measurement of blood glucose concentration is essential for diabetes control. Advances in the field of micro/nanoelectromechanical systems (MEMS and NEMS) now offer unique opportunities in the design of small and ultrasensitive analytical methods. MEMS/NEMS have demonstrated their unique advantages when they are combined with chemistry and biology, including fast, real-time responses. In addition, MEMS and NEMS are small enough to be integrated into other devices. Among the MEMS platforms, microcantilevers (MCLs) have been proven to be an outstanding platform for chemical and biological sensors.[1−16] In several cases, the detection limit of MCL sensors can be as low as femtomolar concentration.[9−10] MCLs can be mass produced through a typical lithograph process and can be readily integrated into a MEMS.

The unique characteristic of MCLs is their ability to deflect due to molecular adsorption or binding-induced change in surface tension. This characteristic of MCLs qualifies them for detecting species in both air and solution. From a molecular point of view, the binding results in electrostatic repulsion[4] or attraction,[9,16] steric effects,[8] and intermolecular interactions[10] that alter the surface stresses on the cantilever. This is achieved by confining the adsorption to one side of the MCL. The key to MCL sensor development is to choose appropriate coatings for identification of chemically specific species.

Modified MCLs can recognize target molecules through specific biological binding, such as binding between antigens and antibodies, or chemical reactions, such as a substrate reaction catalyzed by its corresponding enzyme. These processes cause changes in surface stress of the MCL, which produces the upward or downward bending of the MCL. By recording the deflection magnitude of the microcantilever, the concentration of target biological or chemical species can be measured. Modification of MCL has been realized by self-assembled monolayers,[4,9,11] polymers,[5−7,12] so-gels,[17] and hydrogels,[18−20] etc. Recently, we introduced the nanoassembled layer-by-layer (LBL) approach for MCL modification.[21] The LBL technique, which was developed in 1993,[22] allows the formation of ultrathin, organized films on any surface through alternate adsorption of oppositely charged components, such as linear polyions and enzymes.[23−26] It is a

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simple modification process with nanoscale control of the film thickness. The required component could be positioned at a designed location in the film with nanometer precision. In this paper, we report the detailed investigation of LBL-modified MCL for biosensors.

Many electroenzymatic glucose measurement methods are based on the catalytic activity of glucose oxidase (GOx).[27,28] The foundation of these devices is the oxidation of glucose, which produces gluconic acid, as shown in the following equation.

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{D-gluconic acid} + H_2O_2$$

The reaction results in a decrease in pH, which has been monitored as an indirect measurement of glucose concentration. The detection scheme based on the monitoring of GOx-catalyzed oxidation products of glucose is suitable for continuous monitoring of glucose. The glucose sensing behaviors of GOx-modified microcantilevers and the origin of cantilever deflection are presented.

## EXPERIMENTAL SECTION

**Materials.** In our experiments, we used commercially available silicon microcantilevers (Veeco Instruments). The dimensions of the V-shaped silicon microcantilevers were 180 $\mu$m in length, 25 $\mu$m in leg width, and 1 $\mu$m in thickness. One side of these cantilevers was covered with a thin film of chromium (3 nm) and followed by a 20-nm layer of gold, both deposited by e-beam evaporation. On the uncoated side of the commercial microcantilever was silicon with a 12−19-Å-thick, naturally grown $SiO_2$ layer, which is called "native oxide".

Glucose oxidase (EC 1.1.3.4, type VII-S, from *Aspergillus niger*, 166 500 units/g of solid), $\beta$-D-glucose, D-fructose, D-glactose, D-mannose, sodium salt of 2-mercaptoethanesulfonic acid (MES), and poly(sodium 4-styrenesulfonate) (PSS, MW = 70 000, powder) were used as received from Sigma-Aldrich. Polyethyleneimine (PEI, 14%, MW = 25 000, $\rho$ = 1.043) was a gift from Max Planck Institute. A $10^{-2}$ M MES solution was prepared in water. All other solutions were prepared in a 0.01 M NaCl electrolyte solution (pH = 6.5).

**Deflection Measurement.** The deflection experiments were performed in a flow-through glass cell (Digital Instruments, CA) similar to those used in atomic force microscopy (AFM). The microcantilever was immersed in the 0.01 M NaCl electrolyte solution.

For continuous flow-through experiments, initially, the electrolyte solution was circulated through the cell using a syringe pump. A schematic diagram of the apparatus used in this study was previously reported.[16] A constant flow rate was maintained during each experiment. Experimental solutions containing different concentrations of glucose were injected directly into the flowing fluid stream via a low-pressure injection port sample loop arrangement with a loop volume of 2.0 mL. This arrangement allows for continuous exposure of the cantilever to the desired solution without disturbing the flow cell or changing the flow rate. Since the volume of the glass cell, including the tubing, was only 0.3 mL, a relatively fast replacement of the liquid in contact with the cantilever was achieved. Microcantilever deflection measurements were determined using the optical beam deflection method. The bending of the cantilever was measured by monitoring the position of a laser beam reflected from the gold-coated side of the cantilever onto a four-quadrant AFM photodiode. We define bending toward the gold side as 'bending up"; "bending down" refers to bending toward the silicon side. In case the adsorption occurs on the gold surface, in general, the downward bending is caused by repulsion or expansion of molecules on the gold surface, which is so-called compressive stress; vice versa, the upward bending is caused by attraction or contraction of molecules on the gold surface, which is called tensile surface stress. The cantilever was immersed in the electrolyte solution until a baseline was obtained, and the voltage of the position-sensitive detector was set as background corresponding to 0 nm.

## RESULTS AND DISCUSSION

**Microcantilever Layer-by-Layer GOx Surface Immobilization Process.** The electric charge of a polyelectrolyte in a solution depends on the isoelectric point (pI) of the polyelectrolyte and the solvent. At pH = 6.5, the GOx is a negatively charged polyelectrolyte (the pI of GOx is 4.2).[29]

Since microcantilever bending is generated from adsorption-induced surface stress from one side of the microcantilever, the key surface modification technology is to control the formation of multilayers on only one surface of the microcantilever by choosing appropriate surface materials. In a typical multilayer formation procedure, the aimed target was alternately dipped into a PDDA and a PSS solution, and the process was repeated several times for multilayer formation. When this procedure was applied, multilayer nanoassembly film formation was found on both sides of the cantilever. Recently, we reported a modified multilayer growing process[20] taking advantage of hydrophobic/lipophobic properties of the perfluorocarbon materials. In this method, (tridecafluoro-1,1,2,2-tetrahydrooctyl)triethoxysilane was used to develop a thin perfluorocarbon monolayer on a silicon surface using a typical silicon surface modification procedure, and the polymeric multilayers were found only on the gold surface of the cantilever. However, after a couple of polycation/polyanion cycles, the polymeric multilayer eventually built on the perfluorocarbon surface, especially when there were defects in the monolayer film.[30]

In this work, we found that polyelectrolytes do not stick on a nonmodified gold or silicon surface if the cantilever is rinsed in a fast-flowing water (>100 mL/min), but they build up on a charged surface (e.g., a MES layer) in such severe conditions. Based on this phenomenon, the modified LbL procedure specific for MCL surface modification used in this experiment is as follows: (A) A monolayer of MES was self-assembled on the gold surface of a MCL by immersing the MCL in a 5 mM MES solution for 12 h and then rinsing with EtOH three times followed with deionized (DI) water three times. (B) The MCL was immersed in a PEI solution for 10 min and then rinsed with flowing water at a flow rate faster than 1 m/min for 1 min. The MCL was then immersed

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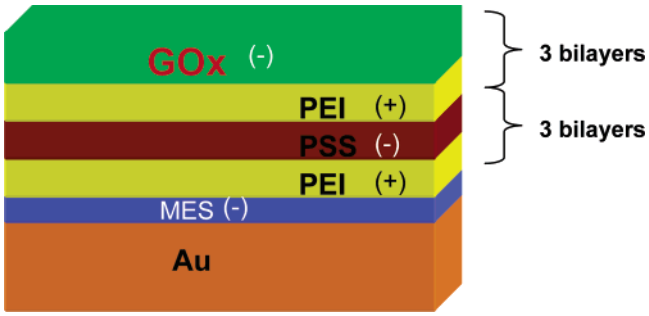


**Figure 1.** LbL nanoassembly with intercalated enzyme on the MCL surface.

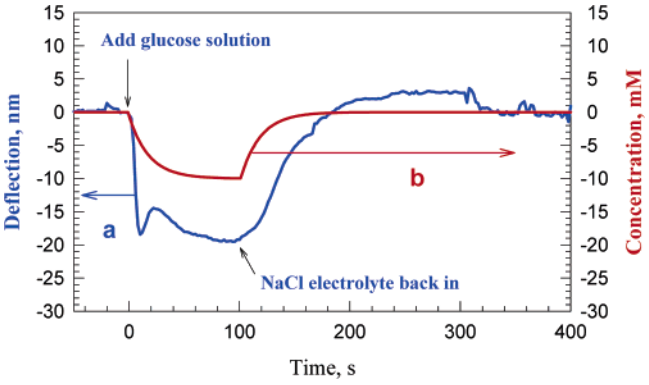


**Figure 2.** (a) Bending response of a multilayer-modified MCL to a 10 mM concentration of glucose in 0.01 M NaCl solution. (b) Deflection of MCL when exposure to different glucose concentrations.

in the opposite polyelectrolyte for 10 min, followed with another rinse with flowing water. C) This cycle was repeated several times until a desired number of multilayers was reached. Figure 1 is a scheme of the LbL assembly.

The method is so efficient that pretreatment with perfluorcarbon film is not necessary. This modified multilayer formation was applied for all the MCLs used in these experiments. In the LbL assembly, the first three bilayers of PEI/PSS were formed that provided a solid base for further enzyme immobilization. Then, three bilayers of PEI/GOx were formed on the top of surface (Figure 1). Each PEI/PSS bilayer was ~1−2 nm in thickness.[24] The reports on PEI/GOx bilayer thickness were conflicted and ranged from 4.4 to 35 nm.[24,29] Eight nanometers is a reasonable number based on the structure of the GOx.[31] Thus, we assume the thickness of the $(PEI/GOx)_3$ layer and the whole assembly was approximately 25 and 30 nm, respectively.

The formation of the polymer multilayer also deflects the cantilever, which was investigated in our former work.[21] In our work for glucose measurement, we set the slightly deflected cantilever as the baseline, i.e., the background corresponding to 0 nm, before glucose injection.

**Flow Rate and Reproducibility.** Figure 2a shows a typical MCL deflection profile when a modified MCL is exposed to a glucose solution at a relatively fast flow rate (72 mL/h). Glucose was added at the marked time. A 2.0-mL aliquot of 10 mM glucose solution was switched into the fluid cell. It took ~100 s for the injected glucose concentration to flow through the fluid cell, and immediately following the glucose, the NaCl

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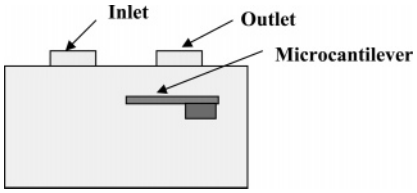
**Chart 1**




electrolyte solution was circulated back through the fluid cell. First, the MCL underwent a downward bending at a maximum of 20 nm in 40 s. When the glucose was replaced by NaCl, the MCL bent backward to its original position (i.e., zero deflection).

In our system, the volume of the fluid cell that held the MCL was ~0.3 mL, and the cell shape is illustrated in Chart 1. The cell was originally filled with NaCl solution. When the glucose solution flowed into the fluid cell, the NaCl solution was replaced by the glucose solution gradually. Similarly, when the NaCl solution was recirculated back into the fluid cell, the glucose solution was replaced by NaCl solution gradually. The glucose concentration change around the MCL was calculated according to eqs 2 and 4, and the profile was shown in Figure 2 to compare with the cantilever deflection profile.

Assuming the injected glucose can flow into the whole cell quickly, eq 1 can be obtained, where $C_G$ is the concentration of

$$\frac{\partial C_t}{\partial t} = \frac{(C_G - C_t)r}{V} \tag{1}$$

glucose injected, $C_t$ is the concentration of glucose in the cell at time $t$, $r$ is the flow rate, and $V$ is the volume of the flow cell.

Integration of the eq 1 gives the following:

$$C_t = C_G - C_G e^{-r/Vt} \tag{2}$$

At ~100 s, when the NaCl solution is recirculated back into the fluid cell, during every $\partial t$ time interval, the molar glucose change in the fluid cell equals the amount of glucose diffusing out ($C_t r$), thus

$$\frac{\partial C_t}{\partial t} = -\frac{C_t r}{V} \tag{3}$$

Integration of the eq 1 gives the following:

$$C_t = C_G e^{-r/Vt} \tag{4}$$

Figure 2 shows that glucose concentration versus time and cantilever deflection versus time profiles matches very well, suggesting that the MCL responded quickly to different glucose concentrations.

At different flow rates, the bending magnitudes of the MCLs at equilibrium were the same at ~20 nm for 10 mM glucose concentration (Figure 3). However, it was obvious that the MCL reached equilibrium faster at higher flow rate following the fast equilibrium of glucose concentration in the flow cell. It was

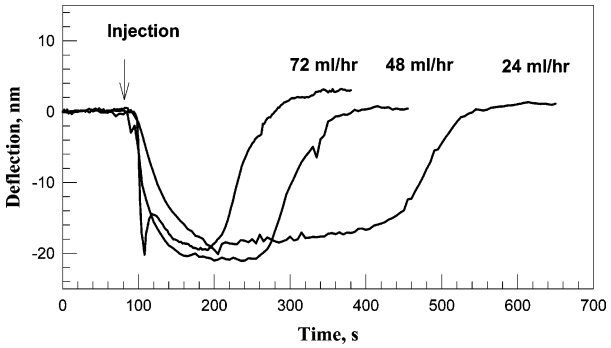



**Figure 3.** Bending response for a $(GOx/PEI)_3$ multilayer-modified MCL to a 10 mM glucose solution in 0.01 M NaCl at different flow rates.

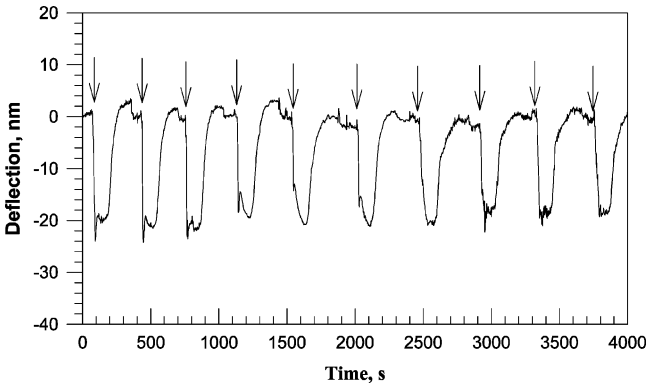



**Figure 4.** Ten replications of bending responses as a function of time for a $(GOx/PEI)_3$ multilayer-modified MCL following injection of a 10 mM glucose concentration in 0.01 M NaCl solution (the injection point is indicated with arrows).

generally thought that faster liquid flow would result in more noise than slower liquid flow. Relatively small flow rates (such as 10 mL/h or slower) were applied exclusively in many previous studies. However, the speculated noise was not observed at a flow rate as high as 72 mL/h. Based on this fact, a fast flow rate would be a better choice for glucose measurement. This is not only because of the shortened analysis time but also due to the elimination of the MCL preequilibrium process. In our previous studies, the MCL had to be kept in the flow cell for a certain amount of time until a perfect baseline was obtained. This may take minutes to hours. Even after a baseline was obtained, some uncertain factors such as the following still caused noise during the measurement: air bubbles, small temperature change, laser intensities variations, and MCL internal stress relaxations. Drift is also a concern when the measurement takes more than 1 h. At a high flow rate, such as 72 mL/h, the whole measurement will take less than 200 s. Statistically, significant noise or drifting does not occur in this time scale. Thus, the time-consuming preequilibrium process is not required, and the sample analysis can be started once the flow system is set.

It was noted that a small peak appears at ~10 s after the sample injection (Figure 3); this occurred only at high flow rates. The peak did not appear consistently as shown in Figure 4. The peak did appear sometimes when a NaCl solution without glucose was injected, but not consistently either. This phenomenon was not fully understood but may be associated with a small turbulence because of the injection or a relaxation process of the MCL.

The reproducibility experiments were conducted by sample-to-sample and MCL-to-MCL measurements. Repeat exposure of a MCL to a 10 mM solution of glucose caused deflection amplitudes similar to that shown in Figure 4. The standard error was within 3%. Exposure of a 10 mM solution of glucose to five different MCLs prepared under the same conditions also caused similar deflection amplitudes and the standard error was within 5%, indicating good sample-to-sample and MCL-to-MCL reproducibility.

Control experiments were performed by exposing a $(PSS/PEI)_6$ modified MCL to a 10 mM solution of glucose. No deflection of the cantilever was observed (figure not shown).

**Relationship between Deflection Amplitudes and the Concentrations of Glucose.** Figure 5 shows the bending response of a GOx multilayer-modified MCL to various concentrations of glucose. The MCL deflection was fully reversible and increased as the concentrations of glucose increased. Since the normal human blood glucose concentration is in the range of 4−6 mM (70−110 mg/dL) and diabetes refers to 8 mM (140 mg/dL) or higher, we focused on measurement of glucose in the range of 1−10 mM. The MCL deflection upon exposure to 20 and 50 mM glucose was also investigated for other applications.[32,33] Figure 6 shows that the deflection amplitudes of the MCL at equilibrium were in direct proportion to the concentrations of glucose injected between the 1- and 50-mM range.

**Selectivity and Lifetime.** It is known that GOx selectively oxidizes glucose.[34] Our results also showed that a GOx multilayer-modified MCL did not deflect upon exposure to other monosaccharides, such as mannose, fructose, and galactose (Figure 7), which agrees with the high selectivity for glucose measurement using GOx-based devices. The stability tests were conducted on GOx multilayer-modified MCLs with 5 months storage in air. The deflection of these over-the-bench MCLs showed a profile and bending amplitude similar to those in Figure 2.

**Deflection Mechanism.** One simple GOx enzymatic reaction mechanism can be expressed as[28]

$$E_{ox} + G \xrightarrow{k_1} GOx{-}G \quad (5)$$

$$GOx{-}G \xrightarrow{K_2} \text{gluconic acid} + E_{red} \quad (6)$$

$$E_{red} + O_2 \xrightarrow{K_3} E_{ox} + H_2O_2 \quad (7)$$

In this mechanism, the $GOx{-}G \rightarrow E_{ox}$ reverse binding was neglected due to the much smaller dissociation constant ($k_{-1}$) compared to $k_1$. Another approximation was made on the second stage of the enzymatic reaction ($E_{red}$ to convert back to $E_{ox}$). This stage may involve the formation of $E_{red}{-}O_2$ complex, decomposition of the complex to $E_{ox}$ and $H_2O_2$, and enzyme conformational change. However, since there has been no direct evidence of the formation of the $E_{red}{-}O_2$ complex,[34] a one-step reaction (eq 7) was used in this work for simplicity. This glucose oxidation reaction is an exothermic reaction with a heat release at −80 kJ/mol.

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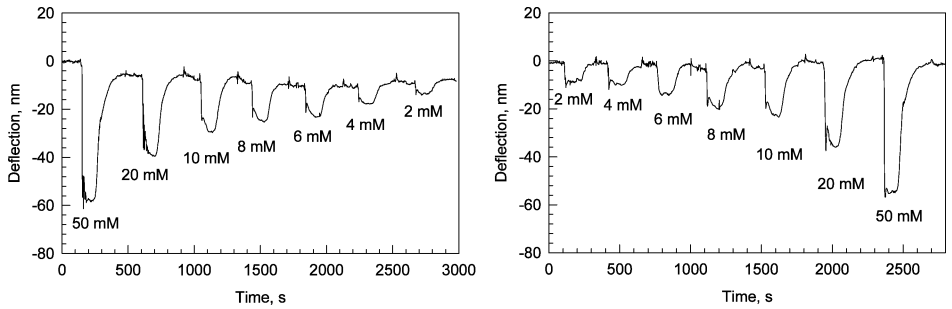



**Figure 5.** Bending response of a $(PEI/GOx)_3$-modified MCL to various concentrations of glucose in a 0.01 M NaCl solution.

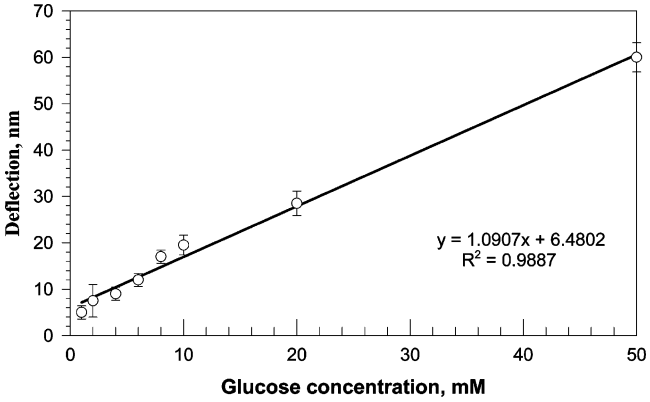
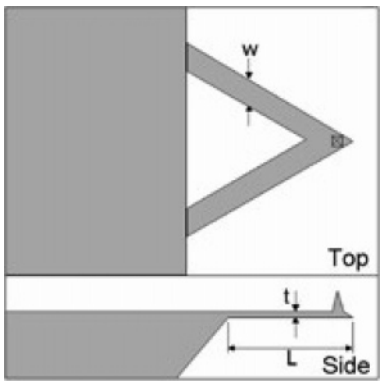



**Figure 6.** Maximum bending amplitude for a $(PEI/GOx)_3$-modified MCL as a function of the change in concentration of glucose.

**Chart 2**




Recently, MCLs for glucose measurement were reported by using typical surface conjugation chemistry[35] and drop coating technique.[36] However, the bending origins are still unknown. To understand the bending mechanism of MCLs, it is necessary to analyze each product or factor that may contribute to the MCL bending based on the glucose oxidation mechanism discussed above. The possible contributions include heat release, pH change, $H_2O_2$ production, and conformational change of the enzymes. Before these contributions are individually analyzed, the overall reaction occurring on the MCL surface is summarized in the following.

The MCLs used in these experiments were triangle silicon MCLs that have a dimension of 180 $\mu$m × 38 $\mu$m × 1 $\mu$m and a layer of 20-nm gold on one surface. (See Chart 2). The MCL surface area was $1.12 \times 10^{-2}$ mm$^2$. Assuming the thickness of the $(GOx/PEI)_3$ multilayer film was ~25 nm, the volume of the $(GOx/PEI)_3$ film (Figure 2) was $\sim 2.8 \times 10^{-13}$ L. It has been reported that the density of the first three GOx/PEI layers was 1.65 $\mu$g/cm$^2$, corresponding to $8.85 \times 10^{-12}$ mol/cm$^2$, i.e., $9.91 \times 10^{-16}$ mol on the MCL surface.[37] Thus, the concentration of the GOx in the multilayer film was calculated to be $3.54 \times 10^{-3}$ M.

At equilibrium, we assume the glucose concentration inside the multilayer film was constant and was equal to the concentration of glucose injected. This assumption was reasonable because the film was only 30 nm thick, the diffusion constant of glucose was high, and the liquid flow rate was as fast as 3 mm/s over the MCL surface.

At equilibrium, the reaction rate in the film can be determined by the Michaelis−Menten equation[38]

$$V = \frac{\partial P}{\partial t} = k_2[E_{ox} - G] = \frac{C_E k_2}{1 + \frac{k_2}{k_1[G]} + \frac{k_2}{k_3[O_2]}} \qquad (8)$$

where P is the product gluconic acid, $C_E$ is the total GOx concentration on the MCL surface, [G] is the glucose concentration (10 mM in Figure 2), and $[O_2]$ is the concentration of $O_2$ in water (1.2 mM).

The kinetic constants vary in different conditions as reported from different groups. An extensive literature survey revealed that the following data were widely accepted for GOx-catalyzed glucose oxidation in solutions: $k_1 = 1.2 \times 10^4$ M$^{-1}$ s$^{-1}$,[39] $K_2 = 800$ s$^{-1}$,[40−42] and $K_3 = 3 \times 10^6$ M$^{-1}$ s$^{-1}$.[42] $k_2/k_1$ is the so-called Michaelis constant ($K_M$). Recently, Calvo's work[43] showed that these kinetic parameters in multilayers were close to those in solutions. Based on these data, we obtained that the reaction rate is $V = 0.36$ M/s.

This reaction rate is very high, which is attributed to the densely packed multilayer of GOx on the surface. It should be noted that this high reaction rate is possible only when the flow

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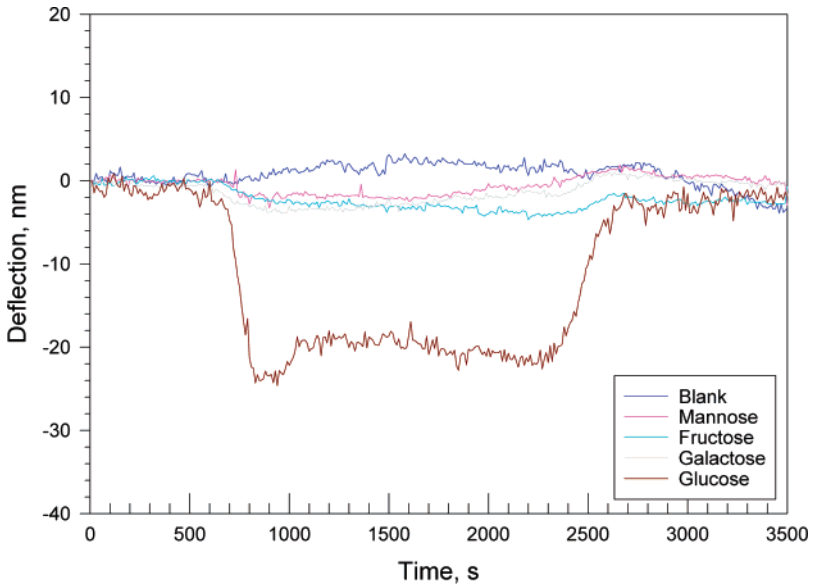


**Figure 7.** Bending responses as a function of time for a $(PEI/GOx)_3$-modified MCL upon exposure to glucose, mannose, fructose, and galactose at a concentration of 10 mM at 4 mL/h flow rate.

rate is high. Otherwise, the glucose concentration will drop significantly during the diffusion in the film; then mass transfer must be taken into consideration.

**A. Temperature Effect.** In a monolayer approach, Thundat et al.[35] concluded that the deflection of MCL was not likely due to thermal energy. Since liquid flow was not taken into consideration in their work, the effect of thermal energy on MCL deflection is further discussed here.

The conversion rate of glucose in the thin, multilayer film calculated above corresponds to $8.85 \times 10^{-12}$ mol/min, and it produces thermal output ($dQ/dt$) at $7.2 \times 10^{-9}$ J/s. At equilibrium or steady state, the temperature difference between the GOx/PEI multilayer-covered surface and the surroundings is constant; i.e., the $dT/dt = 0$. From the one-dimensional heat flow equation, the heat flow can be given by

$$\frac{dQ}{dt} = kA\left(\frac{dT}{dx}\right)_{x\to o}^{-} + hA\left(\frac{dT}{dx}\right)_{x-o}^{+} \qquad (9)$$

where $k$ is the thermal conductivity of silicon (83.5 W/m·K), $A$ is the MCL surface area ($8.51 \times 10^{-3}$ mm$^2$), and $h$ is the convection coefficient of water in these experimental conditions (1020 W/m$^2$·K) that can be calculated from $Nu_x$, assuming the liquid was in laminar flow.[44]

We assume the temperature on the other side (silicon) of the MCL equals the temperature of the surrounding liquid. Again, this assumption is practical especially when the flow is fast. Solving eq 9 revealed that the temperature difference between the GOx/gold surface and surrounding solutions ($\Delta T$) was $7.7 \times 10^{-4}$ K.

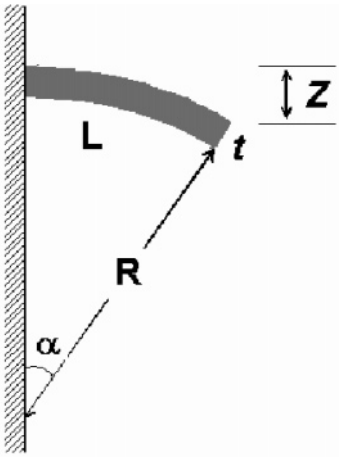


**Figure 8.** Scheme of a MCL under deflection.

According to bimetallic theory of cantilever,[45]

$$\Delta T = \frac{t\left[3(1+m)^2 + (1+mn)\left(m^2 + \frac{1}{mn}\right)\right]}{6(\alpha_1 - \alpha_2)(1+m)^2}\frac{1}{R} \qquad (10)$$

where $\Delta T$ is the temperature difference before and after the bimetallic strip is heated, that is, the temperature difference between the GOx/gold surface and surrounding solutions in our experiments, $t$ is the thickness of the MCL, $m$ and $n$ are the ratio of the thickness and ratio of the modulus of elasticity, respectively, of the gold layer (thickness of 3 nm, modules of elasticity of $0.8\times10^{11}$ Pa) to that of the bottom layer (silicon 1.0 $\mu$m in thickness, modules of elasticity of $1.79\times10^{11}$ Pa), $\alpha_1$ and $\alpha_2$ are the coefficients of expansion for the two materials (gold, $14.2 \times 10^{-6}$ °C$^{-1}$, silicon $2.5 \times 10^{-6}$ °C$^{-1}$), and $R$ is the radius of curvature of the MCL.

(44) $\overline{Nux} = \bar{h}_x x/k_f$, where $Nux$ is the constant (3.73 when the flow cell cut-area from the inlet−outlet is in rectangular shape) and $k_f$ is the thermoconductivity of water (0.60 W/m·K). Calculation showed that $h = 1020$ W/m$^2$·K.

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The scheme of a MCL under deflection is shown in Figure 8. Since the arc angle (α) is very small (because of the small $z$),

$$L^2 + (R - z)^2 = R^2 \tag{11}$$

where $z$ is the deflection amplitude and $L$ is the length of the MCL (180 $\mu$m).

Thus,

$$R = \frac{L^2 + z^2}{2z} \tag{12}$$

Combine eq 12 and 10 results in

$$\Delta T = \frac{h\left[3(1+m)^2 + (1+mn)\left(m^2 + \frac{1}{mn}\right)\right]}{6(\alpha_1 - \alpha_2)(1+m)^2} \frac{2z}{L^2 + z^2} \tag{13}$$

For the $\Delta T = 7.7 \times 10^{-4}$ K generated on the MCL surface in the presence of 10 mM of glucose (Figure 2), the MCL deflection was as small as $7.45 \times 10^{-3}$ nm. Obviously, this deflection is far less than the 20-nm deflection observed in the presence of 10 mM glucose. It is concluded that the contribution from enthalpy change on MCL deflection is negligible.

**B. pH change in the Film.** One of the oxidation products is gluconic acid. It is hypothesized that gluconic acid may protonate the amino groups in PEI, and this results in repulsive multilayer swelling and consequent downward bending of the MCL.

The formation rate of gluconic acid on the MCL surface can be expressed as

$$\frac{\partial P}{\partial t} = V - D_P \frac{\partial^2 P}{\partial x^2} \tag{14}$$

where $P$ is the concentration of gluconic acid, $D_P$ is the diffusion constant of gluconic acid in the GOx/PEI multilayer film, and $V$ is the reaction rate described above.

At equilibrium or steady state, $\partial P/\partial t = 0$, then

$$D_P \frac{\partial^2 P}{\partial x^2} = V \tag{15}$$

No diffusion constant of gluconic acid in the GOx/PEI multilayer film has been reported. However, several reports have shown that an organic compound can diffuse through multilayers at the order of $10^{-8}$ cm$^2$/s and is generally 2 orders of magnitude smaller than its diffusion in water.[46,47] For instance, the diffusion constants for ascorbic acid ($D_A$) and rhodamine B ($D_R$) in multilayers are $4.7 \times 10^{-8}$ [47] and $1.3 \times 10^{-8}$ cm$^2$/s,[48] respectively. According to Stokes−Einstein equation, [48]

$$\frac{D_G}{D_A} = \left(\frac{M_A}{M_G}\right)^{1/3} \tag{16}$$

molecules with similar molecular weight, such as gluconic acid, have a similar diffusion constant. $M_A$ (176 g/mol) and $M_G$ (196 g/mol) are the molecular masses of ascorbic acid and gluconic acid, respectively. The diffusion constant of gluconic acid ($D_G$) is calculated to be $4.5 \times 10^{-8}$ cm$^2$/s.

Thus, solving eq 15 gave $3.6 \times 10^{-5}$ M concentration of gluconic acid on MCL surface at equilibrium. The p$K_a$ of gluconic acid is 3.7. The concentration of protons was then calculated to be $2.9 \times 10^{-5}$ M, i.e., pH = 4.5.

By exposing the GOx/PEI multilayer-modified MCLs to a 0.01 M NaCl solution at pH = 4.5 adjusted by HCl, the pH effect on the multilayer (Figure 9) showed that the pH change did contribute to the MCL bending, but only contributed to half of bending amplitude (10-nm deflection). Furthermore, the MCL response profile to pH was significantly different from that of glucose, which further suggested that pH change was not the only contribution to MCL bending.

**C. Formation of $H_2O_2$.** The formation rate of $H_2O_2$ on the MCL surface also was equal to 0.36 M according to the following equation,

$$\frac{\partial H_2O_2}{\partial t} = k_3[E_{red}][O_2] = \frac{k_3 C_E [O_2]}{1 + \frac{k_3[O_2]}{k_2} + \frac{k_3[O_2]}{K_1[G]}} \tag{17}$$

The faster diffusion constant of $H_2O_2$ (in the order of $10^{-5}$ cm$^2$/s in water) suggested that the concentration of $H_2O_2$ on the surface at equilibrium is less than $3.6 \times 10^{-5}$ M. Control experiments showed that the GOx/PEI multilayer-modified MCLs did not bend upon exposure to $H_2O_2$ at concentration as high as $10^{-2}$ M. Another control experiment was done under oxygen-free conditions, where no $H_2O_2$ will be produced. The bending responses of the MCL were similar in the presence or absence of oxygen (Figure 10).

It is concluded from these results that $H_2O_2$ production does not contribute to the deflection of GOx/PEI multilayer-modified MCL.

**D. Conformational Change of the GOx Enzyme.** GOx is a dimeric protein with a molecular weight of 160 000, containing one tightly bound ($K_a = 1 \times 10^{-10}$ M) flavin adenine dinucleotide (FAD) per monomer as cofactor.[31] The two identical monomers are connected noncovalently via a long but narrow contact area. The corresponding dimensions of the dimer are 70 Å × 55 Å × 80 Å. Each GOx enzyme has two binding sites for substrate.[31]

The conformational changes of GOx have been discussed in the literature.[49−51] Particularly, by using X-ray and other technologies, it was reported that the reduced form of GOx forms several hydrogen bonds between the $\beta$ sheets of the proteins that would contract the enzyme.[49] Furthermore, according to a well-accepted,

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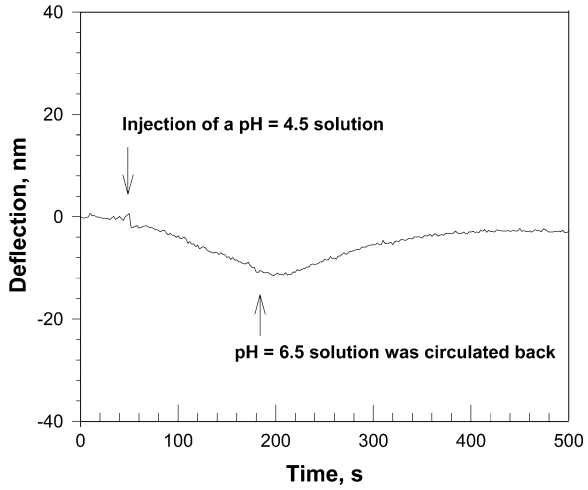


**Figure 9.** Bending responses as a function of time for a $(PEI/GOx)_3$-modified MCL after injection of a 0.01 M NaCl solution with pH = 4.5 at 72 mL/h flow rate. The MCL was preequilibrated in a 0.01 M NaCl solution with pH = 6.5.

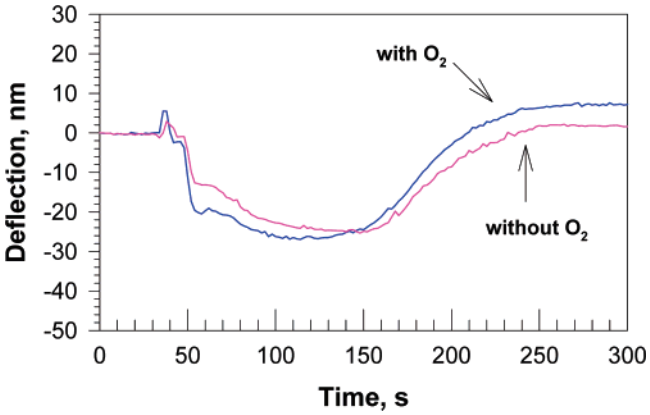


**Figure 10.** Bending responses as a function of time for a $(PEI/GOx)_3$-modified MCL after injection of a 10 mM of glucose in 0.01 M NaCl solution in the absence and presence of $O_2$.

induced-fit theory,[52] the binding process of substrate to enzyme will induce a change in the shape of one or both so that they can fit each other. It is expected that these enzyme conformational changes can result in the surface stress change and the consequent bending response of the MCLs.

To verify this hypothesis, the bending response of the MCL to L-glucose was investigated. It is known that L-glucose could complex with GOx, but no oxidation reaction occurs.[53] Our results showed that the MCL bent to L-glucose (Figure 11) as expected, indicating that conformational change of the GOx enzyme could

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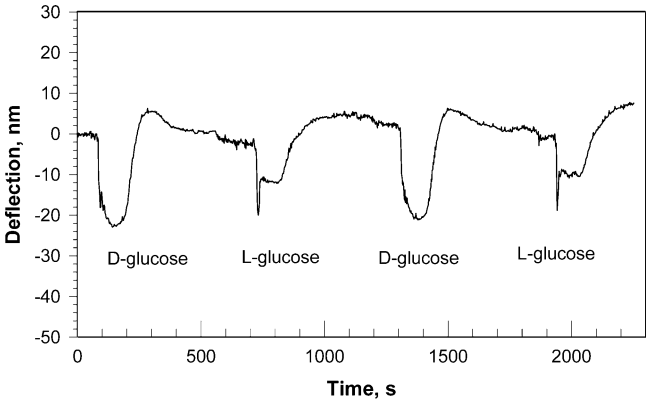


**Figure 11.** Bending responses as a function of time for a $(PEI/GOx)_3$-modified MCL upon exposure to a 10mM solution of D-glucose and L-glucose.

contribute to MCL bending. It is noted that the bending profiles of the MCLs were different in response to L- or D-glucose, indicating different bending origins. The MCL bending in response to L-glucose was generated from pure conformational change of the GOx, but both $H^+$ formation and conformational change of GOx contributed to the MCL bending in the presence of D-glucose. Again, the consistent appearance of the peak immediately after L-glucose injection was not fully understood. The turbulence-induced noise may not satisfactorily explain this phenomenon. A stepwise conformational change of the GOx might contribute to the surface stress change. These phenomena will be further investigated in the future.

## CONCLUSIONS

This paper describes thoroughly a novel enzyme-based microcantilever sensor by using layer-by-layer nanoassembly immobilization technology. The results show that the multilayer approach is a superior approach for MCL surface modifications for enzyme-based biosensor development. The bending mechanism investigation suggests that the conformational change of the GOx enzyme and the protonation to polymers on the MCL are the main reasons for the bending response of the GOx multilayer-modified MCL. The results are not only of technical importance for glucose measurement but also provide a theoretical prediction to enzyme-based MCL biosensors.

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