

# Glucose Detection Based on pH-sensitive Liposomes

Min Kyeong Kang and Jin-Won Park\*

Department of Chemical and Biomolecular Engineering, College of Energy and Biotechnology,  
Seoul National University of Science and Technology,  
232 Gongneung-ro, Nowon-gu, Seoul 01811, Republic of Korea

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pH-sensitive liposomes were employed to amplify the detection of glucose. Glucose oxidase (GOx) covalently immobilized on magnetic particles and pH-sensitive liposomes encapsulating ferricyanide were added to a cyclic voltammeter cell solution in which glucose was distributed. The conversion of glucose into gluconic acid appeared to decrease the pH, which reduced the electrostatic repulsion between the headgroups of weakly acidic 1,2-dipalmitoyl-sn-glycero-3-succinate. The reduction destabilized the liposomes, which released the potassium ferricyanide encapsulated inside them. The effects of the glucose concentration and pH value were investigated. It was necessary to add more than 10  $\mu\text{L}$  of 0.5  $\mu\text{g/mL}$  glucose solution to the 5 mL cyclic voltammeter cell solution to observe a response. The activity of GOx was reversible with respect to the pH change between 7 and 5. The sensitivity of this detection was almost identical to that of comparable techniques such as the use of a field-effect transistor. Therefore, the methodology developed in this study is feasible as a portable and ultrasensitive method.

## 1. Introduction

In energy metabolism, glucose is the most important source of energy for all organisms. Glucose for metabolism is stored in animals as glycogen and circulates as blood sugar. Loss of the control of the glucose concentration leads to diabetes, which has been associated with many medical conditions, including coeliac disease, cystic fibrosis, tuberculosis, and heart disease.<sup>(1–4)</sup> Such complications can result in retinopathy leading to blindness, nephropathy giving rise to renal failure, and peripheral nerve damage with increased risks of extremity ulcers, amputation, and cardiovascular diseases.<sup>(4–8)</sup> Therefore, great effort has been made to achieve the efficient detection and treatment of diseases. Analytical techniques with a detection limit of nM order have been developed for the detection of diseases, including electrochemical or optical sensors.<sup>(9,10)</sup> However, these techniques have a high cost or low portability.

pH-sensitive liposomes include lipid compositions that can be destabilized when the external pH is changed, usually from a neutral or slightly alkaline pH to an acidic pH.<sup>(11)</sup> The liposomes are designed to release their contents, which may be proteins and peptides, antisense

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\*Corresponding author: e-mail: jwpark@seoultech.ac.kr  
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oligonucleotides, plasmids, antibodies, and drugs.<sup>(12–15)</sup> Furthermore, the advantages of the liposomes of biocompatibility, versatility of surface modification, operability of dimensional control, and large-volume internal loading have been utilized.<sup>(16)</sup> Numerous pH-sensitive liposomes have been developed with different mechanisms. The liposomes are mainly prepared with phosphatidylethanolamine (PE), whose structure has a favorable shape for the formation of a hexagonal phase. The destabilization of liposomes requires the inclusion of a weakly acidic amphiphilic molecule such as cholesterylhemisuccinate (CHEMS), phosphatidylserine (PS), or phosphatidylglycerol (PG), which is used to stabilize the liposomes at neutral pH.<sup>(17)</sup> The electrostatic repulsion between the headgroups of these amphiphilics maintains the structure of the liposomes during their interaction. When the liposomes with PE and the weakly acidic amphiphilic molecules are immersed in an acidic environment, they are destabilized.<sup>(18)</sup>

The oxidation of glucose leads to the production of gluconic acid, which reduces the pH value.<sup>(19)</sup> The reduction in pH can be used to stimulate a change in the response. However, since the pKa value of gluconic acid is around 3.7, the change is small, especially in a physiological environment.<sup>(20)</sup> Carbon dioxide is capable of decreasing the pH value, even to less than 5.5. Therefore, pH sensitivity may be considered to amplify the small response. In this work, we aim to develop a portable, rapid, and ultrasensitive method to detect glucose with pH-sensitive liposomes. The pH change from that of glucose triggers the release of the liposomal contents around the electrodes, which are electrons dissociated from the contents at a rate dependent on the applied voltage.

## 2. Materials and Methods

### 2.1 Glucose oxidase immobilization

Glucose oxidase (GOx) was immobilized on magnetic particles through covalent links. A 150  $\mu\text{L}$  aliquot of stock solution (5% w/v) containing 3- $\mu\text{m}$ -diameter particles from Bang Lab (Fisher, IN) was washed three times in 50 mM carbonate buffer (pH 8.2). The particles were coated with 3% (w/v) polyethylenimine (PEI) in 2 mL of 50 mM carbonate buffer (pH 8.2) for 1 h, separated magnetically from the PEI solution, and resuspended by a vortex. The particles were thoroughly washed with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, and 5 mM  $\text{CaCl}_2$  (pH 7.4), and functionalized by reacting 2.5% (v/v) glutaraldehyde in the HEPES buffer solution with PEI coated on them for 45 min. The particles were immersed in 50 mL of HEPES buffer solution (1 U/mL) containing GOx (Sigma, St. Louis, MO) for 3 h. To confirm the immobilization of GOx, the spectra obtained by X-ray photoelectron spectroscopy (PHI 5800, Physical Electronics, Inc., Chanhassen, MN, USA) were utilized. The spectra were acquired from the particles adsorbed physically on a silicon wafer (Sehyung Wafer Tech., Seoul, S. Korea). After the adsorption, the particles on the wafer were treated by a procedure identical to that described above. The concentrations of the injected and unbound enzymes were found using Bradford reagent. In this way, the concentration of the immobilized enzyme was estimated to be about 1.0  $\mu\text{M}$  or 8.0 ng-protein/mg-particle.

## 2.2 Preparation of pH-sensitive liposome

To prepare the pH-sensitive liposome, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-sn-glycero-3-succinate (DPGS) from Avanti were dissolved in a 60:40 ratio (DPPC:DPGS) or in pure DPPC in chloroform. The chloroform was subsequently evaporated at 50 °C under a dry stream of nitrogen to form lipid films on the inside wall of a glass tube. The inside of the glass tube was kept at a low pressure for several hours to remove the last traces of the solvent, and the films were immersed overnight at room temperature in 2 mL of HEPES buffer solution containing 1 mM potassium ferricyanide ( $K_3Fe[CN]_6$ ). The film-suspended solution was obtained through ten 10 min cycles of freezing and thawing with a vigorous vortex, and through the extrusion of two stacked polycarbonate filters with a pore size of 100 nm at 50 °C to form unilamellar liposomes. The liposome solution was transferred to a dynamic light scattering spectrophotometer (ELS-8000, Otsuka, Tokyo, Japan) to measure the diameter of the liposomes, which was found to be distributed between 130 and 170 nm.<sup>(21)</sup>

## 2.3 Cyclic voltammetry experiments

Cyclic voltammetry (CV) experiments were conducted with a CHI660B electrochemical workstation (CH Instruments Inc., Austin, TX). Five milliliters of the HEPES buffer solution in which the GOx-immobilized particles were dispersed uniformly was transferred into a conventional Pyrex glass cell. A Ag/AgCl reference electrode, a Pt wire counter electrode, and a glassy carbon working electrode were immersed in the buffer solution. Then, 10  $\mu$ L of 0.5 mM glucose was injected into the solution in the cell, which was followed by current measurements. The current was measured before and after the injection of the pH-sensitive liposomes described above. The potential was cycled from 500 to  $-200$  mV relative to the reference electrode at a scan rate of 0.05 mV/s. Experiments were repeated three times, and the enzymes were separated from the CV cell solution using magnetic forces.

## 3. Results and Discussion

The immobilization of the GOx was confirmed using X-ray photoelectron spectroscopy (XPS). After each step of the immobilization, the surface was analyzed in terms of elements that have their own binding energy (Table 1).

The relative amount of each element was obtained from the peak distribution for the energy. Prior to any treatment, only peaks of silicon and oxygen were obtained from the surface of the

Table 1  
XPS results for each step of GOx immobilization on magnetic particles.

Element	Silicon oxide (%)	PEI treatment (%)	Glutaraldehyde treatment (%)	GOx immobilization (%)
C 1s	0.1	19.0	23.8	26.7
N 1s	0.1	8.0	6.0	7.4
O 1s	67.5	50.0	48.9	46.5
Si 2p	32.3	23.0	21.3	19.2
S 2p	—	—	—	0.2

silicon wafer. After coating with PEI, the amounts of carbon and nitrogen increased markedly. The increase indicated that the surface was successfully coated with PEI. The changes in the amounts of the elements after the subsequent glutaraldehyde reaction and GOx immobilization were those expected. The results of XPS were consistent with those of previous research.<sup>(22)</sup> The response of the pH-sensitive liposomes was monitored as the pH value was changed from 7 to 5 in steps of 0.5. Then, the concentration of the liposome solution was also varied from 0.5 to 5 mg/mL. Between pH values of 7 and 5.5, no difference in the spectra was observed, while a significant change occurred from pH 5.5 to 5 (Fig. 1).

This trend was identical at all concentrations with precision  $\pm 2\%$ , although the intensities of the measured currents were different. The intensity was proportional to the liposome concentration, as shown in Fig. 2. The intensities were summarized in Table 2.

Since the liposome solution was unstable at a liposome concentration of more than 5 mg/mL, the pH dependence was investigated up to a liposome concentration of 5 mg/mL. This dependence was interpreted with respect to the dissociation constant of DPGS, which was around 5.4.<sup>(23)</sup> A pH of less than 5.4 led to reduced repulsion between the headgroups of the lipids, and we considered that the liposomes started to become destabilized at this pH value. Little shift was observed in the potential of the reduction and oxidation peaks with respect to the concentration of glucose. A shift was found between the reduction and oxidation peaks. Therefore, the process was limited not by kinetics but by diffusion.<sup>(24)</sup> The current responses were continuously monitored after the addition of GOx-immobilized magnetic particle solution, glucose solution, and pH-sensitive liposome solution in this order. Prior to the addition of any of

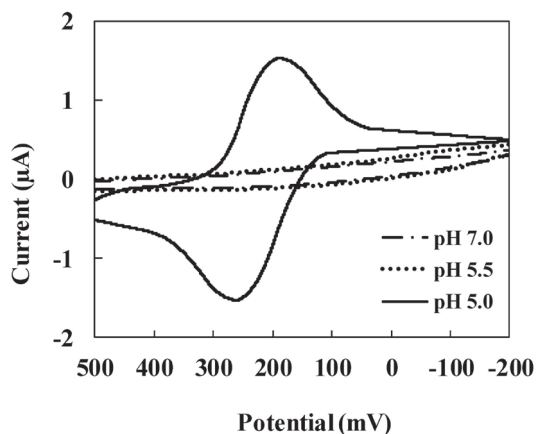


Fig. 1. CV responses for pH-sensitive liposomes encapsulating ferricyanide at different pH values.

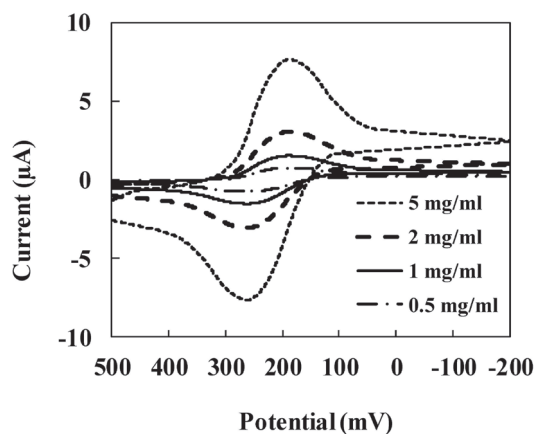


Fig. 2. CV responses for pH-sensitive liposomes encapsulating ferricyanide with different liposome concentrations at pH 5.

Table 2

CV peak for pH-sensitive liposomes encapsulating ferricyanide with different liposome concentrations at pH 5.

	0.5 mg/ml	1 mg/ml	2 mg/ml	5 mg/ml
Current peak ( $\mu\text{A}$ )	$0.77 \pm 0.02$	$1.53 \pm 0.03$	$3.07 \pm 0.03$	$7.66 \pm 0.03$

the solutions, the standard CV curve was confirmed using 1 mM potassium ferricyanide. The liposome concentration in the CV cell solution was 1 mg/mL. The response before liposome addition was almost identical to that of the insulated electrode, even though the electrode surface was untreated.<sup>(25)</sup> Different responses were acquired before and after the addition of the pH-sensitive liposome solution, as shown in Fig. 3.

This result indicated that glucose and GOx by themselves have little involvement in the electrolyte transport. After the addition of the liposome solution, the response increased significantly. Clearly, this increase was caused by the addition of liposomes. However, it was essential to clarify whether the liposome addition itself caused the increase. Therefore, only liposome solution was added to the CV solution without the GOx-immobilized magnetic particles or the glucose. The response with only the liposomes was found to be identical to that of the insulated electrode. For comparison, the addition of GOx-immobilized magnetic particles or glucose solution to the cell was alternately excluded. This comparison was believed to provide insight into the roles of the different added solutions. In particular, when the addition of one of the solutions was omitted, little increase in the response was observed compared with that found prior to the pH-sensitive liposome addition. Therefore, it is concluded that the increase in the response in the presence of GOx-immobilized magnetic particles, the glucose, and the pH-sensitive liposomes is generated by the relation among the GOx, glucose, and liposomes. It was also essential to clarify whether this relation was generated through the change in the pH. Therefore, instead of adding pH-sensitive liposomes, pH-insensitive liposomes made with DPPC only were added. The response of the liposomes with DPPC only was clearly different from that of pH-sensitive liposomes and was almost identical to that when one of the three solutions was not added. Therefore, the relation among GOx, glucose, and the pH-sensitive liposomes that generated the significant increase in the response was triggered by the pH value. After the demonstration of the working principle, the dependence of the response on the glucose concentration was investigated with the addition of 1 mg/mL pH-sensitive liposome solution, with 10  $\mu$ L of the glucose solution injected into the cell at concentrations of 0.1, 0.2, 0.5, and

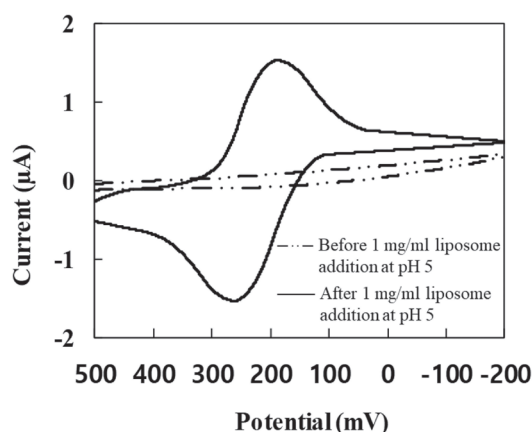


Fig. 3. CV responses before and after the addition of pH-sensitive liposomes to the solution including GOx-immobilized magnetic particles and glucose.

1.0 mg/mL, corresponding to concentrations of 1.11, 2.22, 5.6, and 11.1  $\mu\text{M}$ , respectively. No change in the response was observed at the former two concentrations, while an identical change was observed at the latter two concentrations (Fig. 4).

These results were interpreted with respect to the effect of the pH, which was related to the product formed from glucose. If the concentration of glucose was low, the amount of the reactants involved in the hydrolysis appeared to be insufficient to generate the protons that destabilized the pH-sensitive liposomes. However, it was believed that the liposomes were destabilized at a glucose concentration of 0.5 mg/mL or more. Although more protons may have been generated at higher glucose concentrations, the change in the liposome structure ultimately occurred at a glucose concentration of 0.5 mg/mL. For the response depending on the glucose concentration, the dissociation constant of DPGS was also critical as the result of the change in pH value. The detection limit based on the pH-sensitive liposomes was estimated. Since the response was observed in a 5 mL cell into which 10  $\mu\text{L}$  of 0.5  $\mu\text{g/mL}$  glucose was injected, the detection limit was estimated to be around 10 nM. The proposed technique was found to have comparable sensitivity to the techniques of ELISA and the use of a field-effect transistor (FET).<sup>(26,27)</sup> The reversibility of the GOx activity as a function of the pH value was confirmed to be identical to previous results within the investigated pH range of the cell solution of 7.0 to 5.0.<sup>(28,29)</sup> The selectivity of the detection to other solutes is also important. The detection based on the pH-sensitive liposomes was tested with a mixture containing 1 mg/mL concentrations of glucose, dopamine, and serotonin; these concentrations are much higher than the typical concentrations in body fluid.<sup>(30)</sup> The response of the mixture was little different from that of pure glucose after liposome addition in Fig. 3, while the mixture without glucose showed the same response as that before liposome addition. Analytical techniques with a nM-order detection limit have been developed for the detection, i.e., including electrochemical or optical sensors. These techniques have different advantages. The advantages of using liposome are the simple preparation even for the encapsulating liposomes, the versatility of surface modification, the operability of dimension control, and large-volume internal loading.

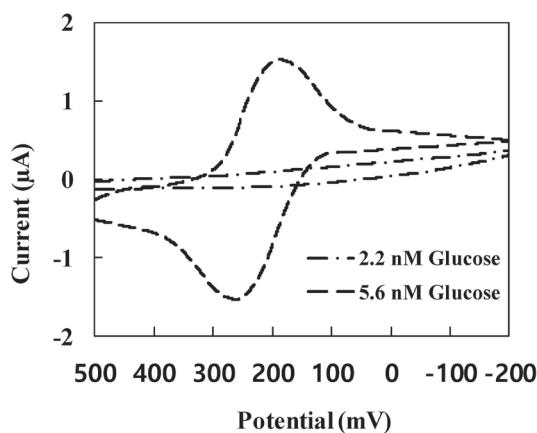


Fig. 4. CV responses for different glucose concentrations with 1 mg/mL pH-sensitive liposome solution.

## 4. Conclusions

In this study, the detection sensitivity of glucose was amplified through the use of pH-sensitive liposomes. The glucose detection was based on the conversion of glucose into gluconic acid. The conversion occurred after the addition of glucose to a CV cell solution in which GOx-immobilized magnetic particles were dispersed. The gluconic acid appeared to decrease the pH, which reduced the electrostatic repulsion between the headgroups of weakly acidic DPGS. The reduction destabilized the liposomes, which released the potassium ferricyanide encapsulated inside them. After the proof of detection concept, the effects of the glucose concentration and pH value were investigated. It was necessary to add 10  $\mu$ L of glucose with a concentration of more than 0.5 mg/mL to the 5 mL CV cell solution to observe a response. The reversibility of GOx was maintained when the pH was changed between 7 and 5. The sensitivity of this detection was almost identical to that of comparable techniques such as the use of a FET. Therefore, the technique developed in this study is feasible as a portable, rapid, and ultrasensitive method.

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## About the Authors



**Min Kyeong Kang** is senior undergraduate student at Seoul National University of Science and Technology. Her research interests are in liposome-based sensors. (dairim2004@naver.com)



**Jin-Won Park** received his B.S. degree from Korea University, Korea, in 1998 and his M.S. and Ph.D. degrees from Purdue University, USA, in 2003 and 2005, respectively. From 2007 to 2010, he was an assistant professor at Gachon University, Korea. Since 2010, he has been a professor at Seoul National University of Science and Technology. His research interests are in biomimetic membranes and their applications. (jwpark@seoultech.ac.kr)