Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research Article

Amplification of urea detection based on pH-sensitive liposomes

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ARTICLE INFO

ABSTRACT

Background: This study aimed to develop an amplification method of urea detection based on pH-sensitive liposomes.
Results: The urease covalently immobilized on the magnetic particles and the pH-sensitive liposomes encapsulating ferricyanide were added to the cyclic-voltammeter cell solution where urea was distributed. The conversion of urea into carbonic acid seemed to induce a pH decrease that caused a reduction in the electrostatic repulsion between the headgroups of weakly acidic 1,2-dipalmitoyl-sn-glycero-3-succinate. The reduction induced the liposomes to release potassium ferricyanide that was encapsulated inside. The effects of urea concentration and pH value were investigated. A specific concentration (0.5 mg/mL) of the urea solution was set to observe the response. The activity of urease was reversible with respect to the pH change between 7 and 5. The sensitivity of this detection was almost identical to the comparable techniques such as an enzyme-linked immunosorbent assay and a field-effect transistor.
Conclusions: In summary, the methodology developed in this study was feasible as a portable, rapid, and sensitive method.

How to cite: Kang MK, Park J-W. Amplification of urea detection based on pH-sensitive liposomes. Electron J Biotechnol 2021;51. https://doi.org/10.1016/j.ejbt.2021.04.005

1. Introduction

Urea is the major end product of nitrogen metabolism in humans, which is one of the most important indicators of possible kidney and liver dysfunction [1]. Early detection of this metabolite is critical because urea concentration can cause heart failure, dehydration, hypovolemic shock, gastrointestinal bleed, and catabolic state [2,3,4]. Furthermore, blood urea nitrogen may reflect several important physiological processes in acute pancreatitis [5]. Therefore, it has been pursued to achieve highly sensitive and specific techniques to detect urea concentration. Analytical techniques including electrochemical, optical, and piezoelectric techniques have been developed for detection [6,7,8]. However, these techniques are usually expensive, less sensitive, or time-consuming. Therefore, it has been a priority to emerge a new methodology to overcome the limitations of the current techniques.

The pH-sensitive liposomes are lipid compositions that can be destabilized when the external pH is changed; usually from a neutral or slightly alkaline pH to an acidic pH. The liposomes are designed to release the contents inside them, which are proteins and peptides, oligonucleotides, antisense, plasmids, antibodies, and drugs [9]. Numerous pH-sensitive liposomes have been developed for each mechanism. The liposomes are prepared mainly with phosphatidylethanolamine (PE) whose structure is a favorable shape for the formation of hexagonal phase. Destabilization requires the inclusion of weakly acidic amphiphilics such as cholesteryl hemisuccinate (CHEMS), phosphatidylserine (PS), and phosphatidylglycerol (PG) that are used to stabilize the liposomes at neutral pH [10]. The electrostatic repulsion between the head groups of these amphiphilics keeps the structure of the liposome interaction. When the liposomes with PE and the weakly acidic amphiphilic are immersed in an acidic environment, they are destabilized [11].

The hydrolysis of urea leads to the production of ammonia and carbonic acid. The ammonia produced remains in an aqueous phase owing to its low vapor pressure; whereas the production of carbonic acid results in pH value reduction [12], which can be used to stimulate a change in the response. However, the pK_a value of carbonic acid is around 3.7, especially under physiological environmental conditions [13]. Carbon dioxide is capable of decreasing

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the pH value even lower than 5.5. Therefore, pH-sensitivity may be considered as amplification for the minute response. In this study, we aimed to develop a portable, rapid, and sensitive method to detect urea with pH-sensitive liposomes. The pH change in urea triggers the release of liposomal contents around the electrodes, which are the electrons dissociated from the contents according to the applied voltage rate.

2. Experiments

Urease was immobilized on magnetic particles through covalent links (Fig. 1). A 150 µL aliquot of stock solution, containing particles of 3 µm diameter from Bang Lab (Fisher, IN), was washed thrice in 50 mM of carbonate buffer at pH 8.2. The particles were coated with 3% (w/v) PEI in 2 mL of 50 mM carbonate buffer at pH 8.2 for 1 h; separated magnetically from the PEI solution, and re-suspended by a vortex. The particles were thoroughly washed with 20 mM HEPES, 150 mM NaCl, and 5 mM $CaCl_2$ at 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 the HEPES buffer solution containing 50 U of urease (Sigma, St. Louis, MO) for 3 h. For the confirmation of the urease immobilization, the spectra of X-ray photoelectron spectroscopy (PHI 5800, Physical Electronics, Inc., Chanhassen, MN, USA) were used. The acquisition of the spectra was performed on particles that were adsorbed physically on a silicon wafer (Sehyung Wafer Tech., Seoul, S. Korea). After adsorption, the particles on the wafer were treated with identical procedures described above. Using the Bradford reagent, the concentrations of the injected and unbound enzymes were found. The concentration of the immobilized enzyme was estimated to be 1.0 µM and 8.0 ng-protein/mg-particle.

For the pH-sensitive liposome preparation, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycero-3-succinate (DPGS) from Avanti were dissolved at 60:40 ratio (DPPC:DPGS) or pure DPPC in chloroform. The chloroform subsequently evaporated at 50°C under a dry stream of nitrogen to form lipid films on the inside wall of a glass tube. The inner side was kept at low pressure for several hours to remove the last traces of the solvent and was 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 formed by freezing and thawing with vigorous vortex every 10 min for ten cycles, and through the extrusion of two-stacked 100 nm pore sized polycarbonate filters at 50°C, uni-lamellar liposomes were formed. The liposome solution was transferred to a dynamic light scattering instrument (ELS-8000, Otsuka, Tokyo, Japan) to measure the diameter of the liposomes, which was distributed between 130 and 170 nm.

Cyclic voltammetry (CV) experiments were conducted with a CHI660B electrochemical workstation (CH Instruments Inc., Austin, TX). The HEPES buffer solution of 5 mL, in which the urease-immobilized particles were dispersed uniformly, was transferred into a conventional Pyrex glass cell. An Ag/AgCl reference electrode, a Pt wire counter electrode, and a glassy carbon working electrode were immersed in the buffer solution. Then, 10 µl of

0.5 mM urea was injected into the solution in the cell, followed by the present measurements. The current was measured before and after injection of the pH-sensitive liposomes described above. The potential was cycled ranging from 500 to – 200 mV relative to the reference electrode at a scan rate of 0.05 mV/s. The whole experiment was repeated thrice, and enzymes were separated from the CV cell solution using magnetic forces.

For the comparison of the above approach, conventional spectrometry was used. A solution identical to the CV experiments was sequentially mixed with the solutions: 0.25 mL of 50 g/L diacetyl monoxime, 0.042 mL of 2 g/L thiosemicarbazide, 0.042 mL of 0.6 g/L Iron(III) sulfate, and 3.33 mL of 50% v/v sulfuric acid. The absorbance of this mixture was monitored at 520 nm. The calibration curve of the absorbance was acquired using solutions with known urea concentration.

3. Results and discussion

Immobilization of the urease was confirmed using XPS. After each step of immobilization, the surface was analyzed in terms of elements that had their own binding energy (Table 1). The relative amount of each element was represented by the peak distribution for energy. Before any treatment, only the peaks of silicon and oxygen were found on the surface of the silicon wafer. After PEI coating, the amount of carbon and nitrogen increased tremendously. This increase indicated that the surface was coated successfully with PEI. These changes led by glutaraldehyde reaction and urease immobilization as the next step, were expected. The results of XPS were consistent with those of the previous research [14].

The response of the pH-sensitive liposome in the absence of both urea and urease was monitored, depending on the change in the pH value from 7 to 5 by 0.5. The concentration of the liposome solution also varied from 0.5 to 5 mg/mL. At pH 7 to 5.5, the difference in the spectra was indistinguishable, whereas a significant change occurred from pH 5.5 to 5 (Fig. 2). This trend was identical at all concentrations, although the intensities of the measured currents were different. The point of change was in the range of pH 5.5 ± 0.05. The intensity was linearly proportional to the concentration of the liposome, as given in Fig. 3. Since the liposome solution was little stable to reproduce more than 5 mg/mL, the pH dependency was investigated up to 5 mg/mL. This dependency was interpreted with respect to the dissociation constant of DPGS, which was around 5.4 [15]. A pH less than 5.4 led to the repulsion between headgroups of the lipids reduced, and then the liposomes were believed to leak potassium ferricyanide (Fig. 4).

The current responses were continuously monitored after each addition. Before all the additions, a standard CV curve was confirmed with 1 mM potassium ferricyanide. The additions were performed with urease-immobilized magnetic particle solution, urea solution, and pH-sensitive liposome solution, respectively. The responses were acquired before and after the addition of the pH-sensitive liposome solution, as suggested in Fig. 5. 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, although non treatment was performed on the
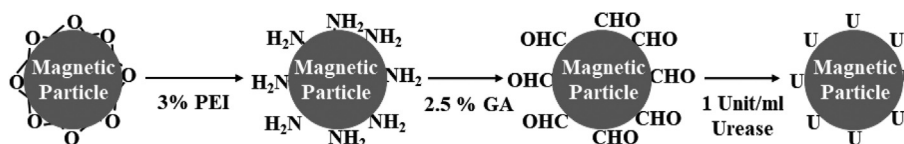

Fig. 1. Scheme used to immobilize urease on magnetic particles, GA and U indicate glutaraldehyde and enzyme.

Table 1

XPS results for each step of urease immobilization on magnetic particles.

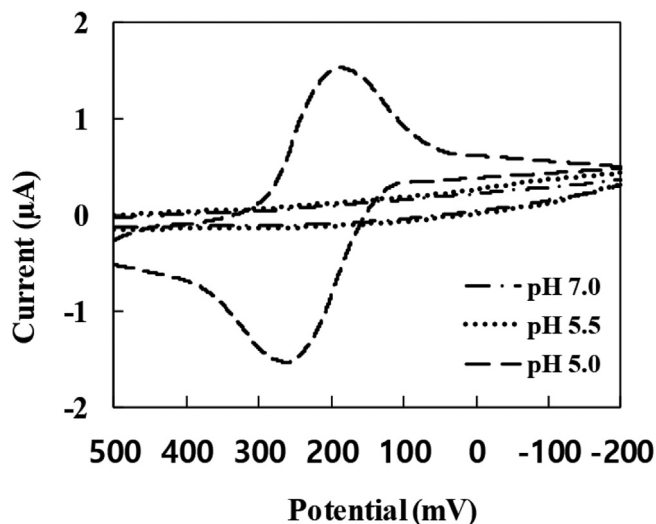|	Silicon oxide	PEI treatment	Glutaraldehyde treatment	Urease immobilization
C 1s	0.1%	19.0%	23.8%	26.7%
N 1s	0.1%	8.0%	6.0%	7.3%
O 1s	67.5%	50.0%	48.9%	46.4%
Si 2p	32.3%	23.0%	21.3%	19.4%
S 2p				0.2%

Fig. 2. Cyclic voltammeter responses only for pH-sensitive liposomes (1 mg/mL) encapsulating ferricyanide at different pH values.
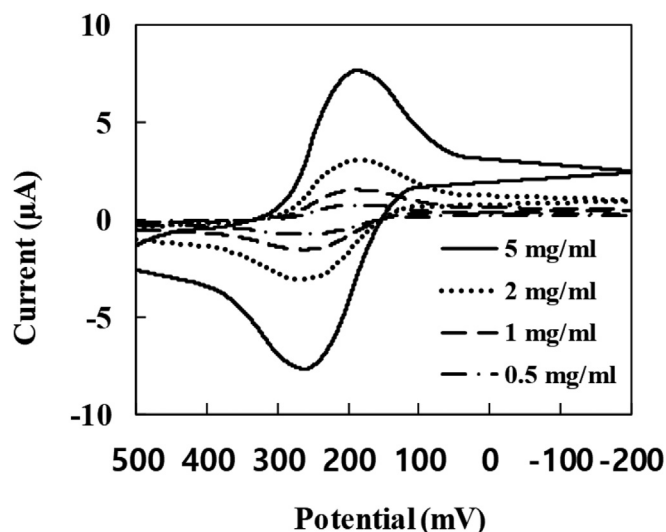

Fig. 3. Cyclic voltammeter responses only for pH-sensitive liposomes encapsulating ferricyanide at different liposome concentrations at pH 5.

electrode surface [16]. This result indicated that urea and urease by themselves are slightly involved in electrolyte transport. After the addition of the liposome solution, the response increased significantly. This increase was caused by liposome addition. However, it was essential to confirm whether the liposome addition by itself caused the increase or not. Therefore, only the liposome solution was added to the CV solution; without the urease-immobilized

magnetic particles and urea. The response with liposomes was found identical to that of the insulated electrode.

For comparison, the additions–the urease-immobilized magnetic particles and the urea solution–were alternately excluded on purpose. This comparison was believed to provide an insight into the role of the additions. As expected, without one of the additions, a small increase in response was observed, and it was found before the pH-sensitive liposome addition. Therefore, it is concluded that the response increase in the presence of the urease-immobilized magnetic particles and urea; and pH-sensitive liposomes appear to be generated by the relation among urease, urea, and the liposome. Furthermore, it was essential to clarify whether this relation was through the change in pH or not. Therefore, instead of pH-sensitive liposomes, only pH-insensitive liposomes made with DPPC were added. The response of these liposomes with DPPC was clearly different from that of pH-sensitive liposomes and almost identical with that one addition that was missing. Therefore, the relation among urease, urea, and the pH-sensitive liposome, generating a significant increase in the response, was based on the pH-triggered.

After the proof of the working principle, the dependency of the urea concentration on the response was investigated at 1 mg/mL pH-sensitive liposome solution. Ten µl of the urea solution was injected into the cell at 0.1, 0.2, 0.5, and 1.0 mg/mL concentrations. Each concentration of these injections was 3.33, 6.66, 16.7, and 33.3 µM, respectively. The ratios of urea to urease were 0.333, 0.666, 1.67, and 3.33. No change in the response was observed at the two former concentrations, whereas an identical change was found at the latter concentrations (Fig. 6). The concentration was validated with conventional spectrometry. These results were interpreted with respect to the pH effect, which was related to the product from urea. If the concentration of urea was low, the reactants for hydrolysis seemed short to generate protons that eventually induced potassium ferricyanide leakage out of the pH-sensitive liposomes. However, at 16 µM or more, it was believed that the liposomes were leaking. Although more than 16 µM might generate more protons, the change in the liposome structure ultimately occurred equally with 0.5 mg/mL. For the response on the urea concentration, the dissociation constant of DPSG was also critical as the results, with respect to the change in pH value.

The sensitivity of detection based on pH-sensitive liposomes was estimated. Since the response was observed at 5 ml cell to which 10 µl of 0.5 µg/mL urea was injected, the sensitivity was estimated to be around 10 nM concentration. The comparable techniques; FET and piezoelectricity were found to be similar (Table 2) [6,8]. The reversibility of the urease activity on the pH value was confirmed to be identical with the previous results, in the range of 7.0–5.0; where the pH of the cell solution was continuously changed [17]. The selectivity to other neurotransmitters was also important. The detection based on the pH-sensitive liposomes was tested with a mixture of urea, dopamine, and serotonin. In the mixture solution, each component was of 1 mg/mL concentration; much higher than the typical concentration [18]. The response of the mixture was slightly different from that of pure urea, when the mixture was without urea.

Fig. 4. Schematic diagram of the phenomena after urea, urease, and pH-sensitive liposome injection – decomposition of urea and the effect of proton production.

Fig. 5. Cyclic voltammeter responses before and after the addition of the pH-sensitive liposomes (1 mg/mL) into the solution including the urease-immobilized magnetic particles (10 μM) and the urea (33.3 μM).

Fig. 6. Cyclic voltammeter responses on the urea concentration at 1 mg/mL pH-sensitive liposome solution.

Table 2
Comparison of sensitivity for comparable techniques.

	CV based on the pH-sensitive liposomes	FET	Piezoelectric
Sensitivity	10 nM	10 nM	10 nM

4. Conclusion

In this study, the detection of urea was amplified through pH-sensitive liposomes. Urea detection was based on the conversion of urea into carbonic acid. The conversion occurred after the addition of urea into the CV cell solution where the urease-immobilized magnetic particles were dispersed. Carbonic acid seemed to induce the pH decrease that caused the reduction in electrostatic repulsion between the headgroups of weakly acidic DPGS. The reduction induced the liposomes to release potassium ferricyanide encapsulated inside.

After proving the detection concept, the effects of urea concentration and pH value were investigated. More than 0.5 mg/mL urea solution with 10 μl addition into a 5 ml CV cell solution was set to observe the response. The reversibility of urease was kept with respect to the pH change between 7 and 5. The sensitivity of this detection was almost identical to comparable techniques such as an enzyme-linked immunosorbent assay and a field-effect transistor. Therefore, the technique developed in this study is feasible as a portable, rapid, and sensitive method.

Financial support

Conflict of interest

The authors declare no conflicts of interest.

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