ACS OMEGA

http://pubs.acs.org/journal/acsodf

Article

Urea Biosensor Based on a CO_2 Microsensor

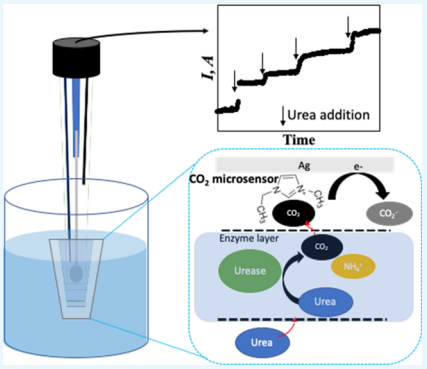
Deby Fapyane, Dmitriy Berillo, Jean-Louis Marty, and Niels Peter Revsbech*

Cite This: *ACS Omega* 2020, 5, 27582−27590

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Urea sensors based on electrodes in direct contact with the medium have limited long-term stability when exposed to complex media. Here, we present a urea biosensor based on urease immobilized in an alginate polymer, buffered at pH 6, and placed in front of a newly developed fast and sensitive CO_2 microsensor, where the electrodes are shielded by a gas-permeable membrane. The CO_2 produced by the urease in the presence of urea diffuses into the microsensor and is reduced at a Ag cathode. Oxygen interference is prevented by a Cr^{2+} trap. The 95% response time to changes in urea concentration was 120 s with a linear calibration curve in the range 0−1000 μM and a detection limit of 1 μM. The Ni^{2+} cofactor to improve sensor performance was continuously supplied from a reservoir behind the sensor tip. The stability of the urea sensor was optimized by the addition of bovine serum albumin as a stabilizer to the urease/alginate mixture that was cross-linked with glutaraldehyde and Ca^{2+} ions. This immobilization strategy resulted in about 70% of the initial urea sensor sensitivity after two weeks of continuous operation. The sensor was successfully tested in blood serum.



INTRODUCTION

Urea is a dominant final metabolite of nitrogenous compounds in humans and animals (mammals), and blood serum urea concentration is used as a marker for liver and kidney function.[1] The urea concentration in human plasma varies between 1 and 10 mM depending on the individual diet and condition, whereas the concentration can be 50-fold higher in urine.[1] In addition to the medical field, urea analysis is also important in the agricultural and food industries and in environmental monitoring. Urea is used widely as a nitrogen fertilizer[2] in farming. In the food industry, urea released by yeast cells by fermentation processes must be regulated as urea forms carcinogenic ethyl carbamate in the presence of ethanol.[3] Urea also plays a key role in the marine nitrogen cycle, with excretion by marine bacteria and animals as the source and phytoplankton consumption as major sink.[4] Long-term urea exposure to aquatic ecosystems leads to eutrophication, ammonia release after urea hydrolysis by urease, and accumulation of the oxidation products of ammonia such as nitrate, nitrite, and N_2O.[5] Hence, reliable analytical tools for urea quantification are essential.[6]

A variety of techniques have been developed for determination of urea, by direct or indirect detection. Direct determination of urea includes chromatographic[7] and colorimetric[8] methods that are reliable and have been widely used for general monitoring. However, these techniques require complicated sample preparation and/or sophisticated equipment, making them unsuitable for online or onsite monitoring. Direct detection by urea-oxidizing electrodes is possible using Ni-based catalysis.[9−12] Linear calibration curves over considerable concentration ranges have been obtained by such direct oxidation, and the sensors exhibited excellent long-term stability by storage in air or defined buffer. Glucose was found to interfere, and Ni catalysts have actually been thoroughly studied for use in nonenzymatic glucose sensors.[13] The sensors were tested in media such as tap water and >100 times diluted urine. Apparently, there are no reports of performance by long-term exposure to proteins and other electrode-inactivating substances.

Indirect methods for urea quantification refer to the enzymatic degradation of urea by urease prior to hydrolysis product detection,[7] and such a technique is the focus of the present study. Urease is a metalloenzyme that is commonly extracted from plants such as *Canavalia ensiformis* (Jack bean)[14] and *Glycine max* (soybean) and bacterial sources such as *Bacillus pasteurii*. It is highly selective in catalyzing the hydrolysis of urea to ammonium (NH_4^+) and carbon dioxide (CO_2) or its ionic forms (HCO_3^- or CO_3^{2-})[14] depending on the solution pH.[15] The metals at the active sites in ureases are necessary for activating urea and water into reaction through a hydroxide-bridged dinuclear nickel complex.[16] Because of its specificity and selectivity to urea, urease is being integrated as a biomolecule recognition moiety in urea biosensors.

The development of electrochemical urease-based biosensors has been the topic of several studies. Biosensors based on

Received: August 27, 2020
Accepted: October 2, 2020
Published: October 19, 2020

https://dx.doi.org/10.1021/acsomega.0c04146
ACS Omega 2020, 5, 27582−27590

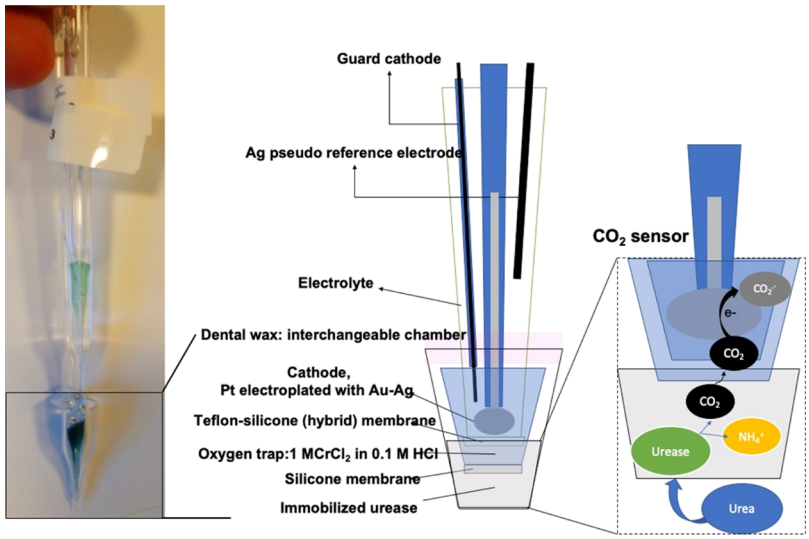


Figure 1. Photograph (left) and schematic representation of the urea microsensor based on the CO_2 microsensor. The CO_2 produced from urea hydrolysis diffuses through the gas permeable membranes of the CO_2 microsensor and is detected through reduction at a Ag cathode. The analyzed medium should be at a defined pH around 6 to ensure that most inorganic carbon is present as CO_2. All data presented were obtained in 10 mM citrate buffer, pH 6. Photograph by Fapyane.

electrochemistry offer potential for high selectivity−sensitivity, low detection limit, possibility for miniaturization, and fast response. Hitherto, there are a number of publications focusing on electrochemical urea detection using polyalanine or glutamate dehydrogenase-assisted NH_4^+ sensing.[17−19] Direct cathodic ammonia/ammonium oxidation[20] and potentiometric ammonium electrodes[21] have also been applied. However, an NH_4^+-sensing transducer is affected by the presence of NH_4^+ and possibly other cations in the electrolyte. Furthermore, interference from easily oxidized small molecules may lead to analytical errors. An alternative to specific determination of NH_4^+ is to measure the current originating from binding of the ions formed by urease action on a functionalized electrode surface,[22] but as might be expected, the performance of such a sensor is highly dependent on the ionic composition of the analyzed medium.

The first product of urease hydrolysis is carbamic acid which is highly unstable and is further hydrolyzed into ammonium and water. Some hydrazine is formed as a side product, and direct anodic oxidation of this hydrazine can be used as a detection mechanism.[23] Hydrazine is formed by reaction between two short-lived carbamic acid molecules, and only relatively high urea (millimolar) concentrations could thus be quantified by this approach.

The urease-based sensors described above are all based on electrodes in direct contact with the analyzed liquid, and they are thus prone to inactivation by medium constituents such as proteins. No reports of long-term exposure to complex media such as blood serum could thus be found. A solution to this challenge is to apply CO_2 monitoring based on Severinghaus-type CO_2 sensors.[24−26] Severinghaus-type sensors are based on diffusion of dissolved CO_2 through a gas permeable membrane, resulting in a pH change in a buffer behind this membrane.[27] Electrode fouling substances such as proteins cannot penetrate the polymer membrane, and there should thus be basis for better long-term stability in complex media. However, Severinghaus sensors are characterized by unstable calibration curves and poor performance at low CO_2, resulting in suboptimal performance of urea biosensors based on this technique.[28]

We propose a novel urea biosensor based on an amperometric CO_2 microsensor[29] that is fast responding and sensitive. The urease enzyme is integrated into the sensor by entrapment, stabilization, and cross-linking in the alginate polymer and inserted in a glass chamber in front of the CO_2 microsensor. The urease mixture is buffered at pH 6 to ensure that most of the CO_2 produced by urea hydrolysis is in the gas form (not HCO_3^- or CO_3^{2-}) to be able to diffuse through a gas permeable membrane and into the CO_2 microsensor. The effects of the enzyme amount, type, and concentration of supplied metal cofactors and urease immobilization methods on sensor sensitivity, response time, and stability are elucidated. The novel urea sensor only requires a simple sample preparation for urea measurement in blood plasma.

RESULTS AND DISCUSSION

CO_2 Microsensor Performance. The most important key parameter for successful urea biosensor performance, according to the scheme outlined in Figure 1, is the performance of the CO_2 microsensor[29] itself. A typical CO_2 microsensor 90% response time is 60 s. The linear range of a sensor optimized for high sensitivity is about 0−500 μM CO_2. When three sensors were long-term tested, they exhibited no noticeable change in zero current (I_o) and CO_2 sensitivity during 5 months of continuous polarization (Figure S1).

Effect of the Urease Amount. Successful performance of our proposed CO_2 microsensor-based urea microsensor is dependent on complete hydrolysis of urea diffusing into the alginate/urease matrix so that urea is depleted before it reaches the CO_2 microsensor tip. Similar biosensors, where NO_3^- or NO_2^- is converted to N_2O that is detected with a Clark-type microsensor, also exhibit linear response to the analyte when the ions are transformed before they reach the gas sensor.[30,31] Several parameters such as the relative enzyme activity, the concentration of the enzyme, the length of the reaction chamber, the enzyme stability, and the immobilization

https://dx.doi.org/10.1021/acsomega.0c04146
ACS Omega 2020, 5, 27582−27590

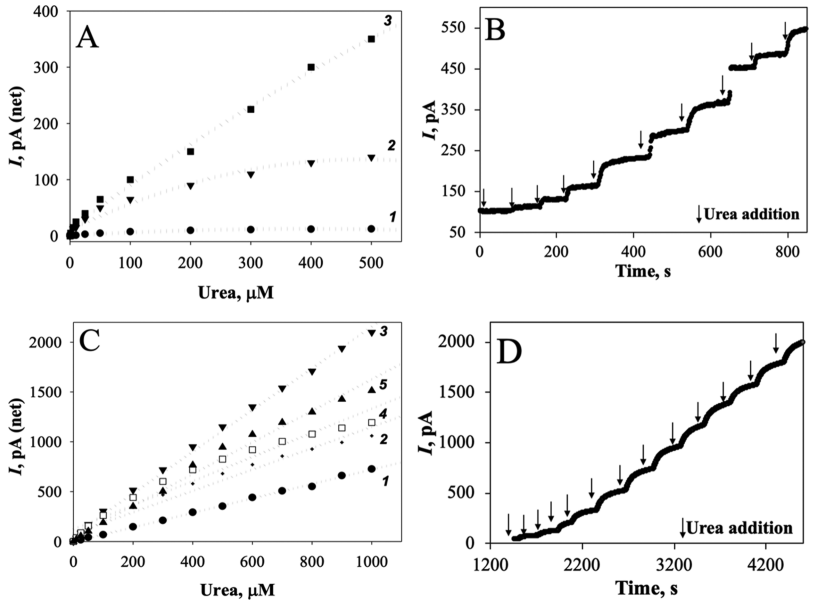


Figure 2. Calibrations under various conditions of urea biosensors with urease in 5% alginate in front of the CO_2 microsensor. All experiments were conducted at 20 °C and pH 6. (A) Calibration curves in the presence of 3 (1), 30 (2), and 220 (3) mg urease/mL. (B) Raw data for calibration with 220 mg/mL urease. (C) Calibration curves of the sensor with 220 mg urease/mL in the presence of 0 (1), 1 (2), 10 (3), 25 (4), and 50 (5) mM Ni^{2+}. (D) Raw data for calibration with 220 mg/mL urease in 5% alginate, supplied with 10 mM Ni^{2+}. The urea sensor tips were 80 μm.

efficiency all play important roles in the urea microsensor performance.

Stability of the enzyme layer was optimized by entrapping or cross-linking the urease in alginate in the presence of Ca^{2+} ions. The Ca^{2+} ions induce polymerization in alginate solution by bridging two COO^- groups, forming a solid alginate gel. The Ca^{2+} ion itself was shown to have a beneficial effect on the urease activity as it may mimic the cofactor function of Ni^{2+} (measured with Berthelot reaction, Figure S2A). The enzyme layer thickness can be altered by regulating the space between the tips of the enzyme chamber and CO_2 microsensor. We routinely made the sensors with 200 μm distance between the CO_2 microsensor tip and the enzyme chamber tip to ensure complete hydrolysis of urea up to 1 mM. As shown in Figure S3, a 100 μm distance, actually, also resulted in linear calibration up to almost 1 mM. Longer reaction chambers (>200 μm) resulted in impractically long response times.

In order to completely hydrolyze all the in-diffusing urea, the amount of enzyme in the enzyme layer needs to be high. When a low amount of urease was used (3 mg/mL), the sensor signal was low and nonlinear. When the urease concentration was increased from 3 to 30 mg/mL, the response was higher but again nonlinear. A linear correlation of sensor response to urea concentration in the range 0−500 μM was approached (r = 0.998) when urease concentration was increased to 220 mg/mL in the enzyme chamber (Figure 2A). The 95% sensor response time was 120 s after urea addition with a sensitivity of 0.6 pA/μM (tip size 80 μm) (Figure 2B). A 220 mg/mL urease concentration was used for all subsequent tests.

Effect of Metal Cofactor Supply and Its Concentration. Urease is the only known metallohydrolase enzyme that utilizes nickel (Ni^{2+}) as a cofactor. The reaction catalyzed by the binickel sites in urease is the hydrolysis of urea to form NH_4^+ and CO_2.[32,33] The proposed mechanism of urea hydrolysis at the active site involves production of the hydroxide ion at the metal center, activation of the substrate coordination with both metal ions and nucleophilic attack of the substrate, and producing NH_4^+ and carbamic acid (NH_2CO_2H), which is spontaneously decomposed to form one more molecule of NH_4^+ and CO_2.[16,34] The hydrolysis of carbamic acid induces a pH increase which may have a negative impact on the urease activity. An increase in pH would also lower the efficiency of inorganic carbon detection by our sensor design, and a buffering of the analyzed solution at relatively low pH is thus essential.

Urease is a very stable enzyme that keeps its conformation even by loss of the Ni^{2+} cofactor, but the activity is lowered.[33] Thus, Ni^{2+} availability plays an important role in the urease-catalyzed hydrolysis reaction. We utilized Jack bean urease which has eight active centers, resulting in a demand of 16 Ni^{2+} per urease molecule (Toyobo enzymes). By supplying the mixture with 1 mM Ni^{2+}, the sensor signal was almost doubled as compared to no added Ni^{2+} (Figure 2C(1,2)). By adding 10 mM Ni^{2+}, the sensor response to urea concentration from 0 to 1000 μM achieved almost perfect linearity (Figure 2C(3), R^2 = 0.999, and Figure 2D) with approximately three times higher sensitivity (2 pA/μM urea), as compared to no Ni^{2+} (Figure 2C(1)). The high effect of Ni^{2+} addition may be explained by loss of Ni^{2+} during enzyme purification, resulting in lowered activity. Nickel addition thus apparently reactivated the inactive urease, achieving a higher urease hydrolysis rate. The highest activity was achieved when the enzyme was exposed to the Ni^{2+} solution before mixing with alginate. Excessive Ni^{2+} exposure to the urease mixture was, however, detrimental to sensor performance, as shown by the lowered activity at 25 and 50 as compared to 10 mM Ni^{2+} (Figure 2C(3−5)).

Mn^{2+} and Co^{2+} have also been proven to activate urease but at lower efficiency.[35] The same concentration (10 mM) of Mn^{2+} and Co^{2+} ions was able to increase the sensor performance (220 mg/mL urease in 5% alginate) by 20−30% as compared to no cations added. However, the sensor signals with Mn^{2+} and Co^{2+} were considerably lower than those

https://dx.doi.org/10.1021/acsomega.0c04146
ACS Omega 2020, 5, 27582−27590

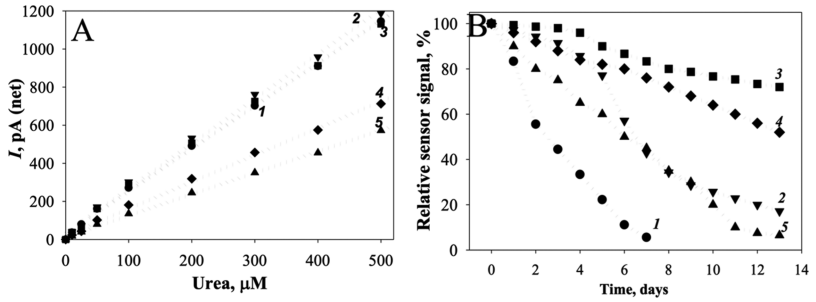


Figure 3. (A) Effect of the urease immobilization strategy on urea sensor response. The urea sensor tip sizes were 80 μm. (B) Effect of the urease immobilization method on relative sensor response during 14 days (signal at $day_x/day_0 \times 100\%$) by the addition of 25 μM urea. The numbers in graphs A and B refer to the same immobilization mixtures: (1, circle) urease/alginate, (2, downward triangle) urease/alginate/BSA, (3, square) urease/alginate/BSA/GLU, (4, diamond) urease/alginate/BSA/GLU/CHI, and (5, upward triangle) urease/alginate/GLU/CHI. BSA: bovine serum albumin, GLU: glutaraldehyde, and CHI: chitosan. All the sensors were freshly made before test.

Table 1. Urease Stabilization/Immobilization Strategies and Urease Mixture Materials

material	final concentration	entrapped	entrapped−stabilized	cross-linking 1	cross-linking 2	cross-linking 3
urease	220 mg/mL	v	v	v	v	v
Ni^{2+}	10 mM	v	v	v	v	v
BSA	200 mg/mL		v	v	v	
chitosan	0.05%				v	v
glutaraldehyde	0.5%			v	v	v
alginate	5%	v	v	v	v	v

when using Ni^{2+} (Figure S2B(2−4)), and therefore, we activated the enzyme with Ni^{2+} in all subsequent experiments.

Effect of Enzyme Stabilization and Immobilization on Urea Sensor Performance. Jack bean urease/urea amidohydrolase (α-urease, EC 3.5.1.5) is a hexameric protein (M_w: 480−545 kDa), consisting of six identical subunits, each with a molecular mass of 91 kDa.[36] In the presence of urea or by the addition of polyalcohols, hexameric urease dissociates to its active dimer form.[37] Native urease (nonmodified enzyme) can dissociate into the active, folded dimer or monomer in water and eventually turn into inactive monomer aggregates. The addition of amphiphilic molecules such as sodium dodecyl sulfate or bovine serum albumin (BSA) stabilizes the active monomer by bridging the hydrophobic residue in the proximity of the redox center to water, preventing the change of conformation into an inactive monomer form.[37]

The dissociation mechanism of native urease in the aqueous medium gives us hints about how to design a stabilization and immobilization procedure. Immobilization of urease limits the mobility of its subunits, and they thus cannot migrate freely after dissociation. The immobilization may thus help to keep the dimer conformation, whereas free urease monomers dispersed in water can change their conformation to nonactive aggregates.[38] Various enzyme immobilization techniques have been developed. Surface adsorption and polymer-based entrapment are simple and effective, but enzyme leaching may result in rapid loss of activity.[39] Covalent attachment and cross-linking can prevent leaching and be effective and durable, but there is a high risk of decreasing the enzyme activity by disturbed enzyme conformation.[39] The addition of compounds with suitable functional groups for covalent bonding has been proposed to lower the detrimental effects on enzyme activity by lowering the covalent bonding directly to the enzyme.[39,40] Support protein such as BSA or polymers such as chitosan and alginate are used for cross-linking to enzymes by the use of glutaraldehyde,[41,42] and for urease, it was shown to result in a retained activity of more than 50%[40] as compared to the free enzyme, with some activity still being present after a few weeks.[35]

In this study, optimal immobilization of urease in the enzyme chamber is very important for sensor performance and stability. We have screened several polymers in addition to alginate such as poly(ethylene glycol) dimethacrylate (PEGDMA), chitosan, and poly(vinylalcohol)-*N*-methyl-4(4′-formylstyryl)pyridinium methosulfate acetal (PVA-SBQ) for urease immobilization. It was shown that the combination of alginate/Ca^{2+} ions preserve urea activity to a higher degree than UV curing-based polymer combinations such as PEGDMA/UV, PEGDMA/chitosan/UV, or PVA-SBQ/UV (Figure S4A).

The entrapment of urea in alginate/Ca^{2+} may preserve the urease activity, but it may not prevent enzyme leaching. Hexameric urease has a size of 10−13 nm, depending on its source,[43] but it dissociates to its dimer with a size of 2−4 nm. The cross-linked alginate pore size is between 1 and 10 nm depending on the alginate concentration,[44] making it possible for entrapped urease to leach from the sensor tip. Thus, strategies for firmer urease immobilization such as carbodiimide bonding and cross-linking with glutaraldehyde were investigated. Immobilization of urease in alginate through carbodiimide bonding (facilitated by EDC/NHS) was shown to diminish urease activity by more than 90% (Figure S4B), as might be expected from a previous report stating that this process affects urease conformation and activity.[39] Hence, additional materials such as BSA (200 mg/mL) as a stabilizer and chitosan (0.05%) as an amine-containing support polymer were added to the urease/alginate mixture. The effects of different additives and enzyme immobilization methods (entrapment or cross-linking) were evaluated through urease activity using a colorimetric method (Berthelot) and urea sensor performance.

BSA addition to the urease/alginate/Ca^{2+} mixture had no significant effect on urease activity and sensor sensitivity. When glutaraldehyde was added as a cross-linking agent to the

https://dx.doi.org/10.1021/acsomega.0c04146
ACS Omega 2020, 5, 27582−27590

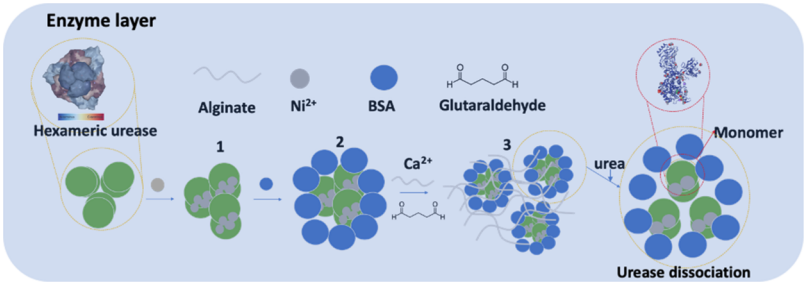


Figure 4. Urease immobilization strategy. Hexameric urease was supplied with its Ni^{2+} cofactor and mixed with BSA. Alginate was then added to the mixture of urease, Ni^{2+}, and BSA and subsequently polymerized with Ca^{2+} ions and glutaraldehyde. In the presence of urea, the hexameric urease dissociates into its active monomers. Urease entrapment in alginate prevents the urease monomer from leaching.

Table 2. Comparison of Electrochemical Urea Biosensor Performance Parameters[a]

urease immobilization strategy	sensor type	measured analytes	response time, min	LOD, μM	relative stability[b]	refs
immobilized urease on the Teflon membrane	amperometric	NH_4^+	5	500	90% in 7 days	46
urease cross-linked in the polyaniline–Nafion membrane with glutaraldehyde	voltammetric	NH_4^+	5	5	N/A	20
urease entrapment in the nanostructure	amperometric	NH_4^+	0.03	500	80% in 7 days	18
urease entrapped in the polyion complex	amperometric	NH_4^+	0.03	50	1 day	48
urease entrapped in PVA-HMDI	carbon-based electrode	carbamic acid	1–2	500	60% in 7 days	47
urease/BSA cross-linked with glutaraldehyde on the gas-permeable membrane	Severinghaus, potentiometric pH sensor	CO_2	12–30	15	65% in 10 days	26
urease/BSA in alginate, cross-linked with glutaraldehyde and Ca^{2+} ions	amperometric CO_2 microsensor	CO_2	2	0.94	70% in 14 days	our study

[a]The stability recorded was by continuous polarization for this study and by shelf storage for the other studies. [b]Response at $day_x/day_1 \times 100\%$.

urease/BSA/alginate and urease/BSA/alginate/chitosan mixtures, the urease activity decreased approximately by 10% for both cases, suggesting only minor harm to the urease (Figure S4C), as also shown by urea sensor performance (Figure 3A(3)). BSA and chitosan may both serve the same function as the amine group donor to make urease less affected during the cross-linking process. However, BSA may also play a role as a stabilizer because of its amphiphilic properties (or nonionic surfactant-like properties),[45] which may protect the hydrophobic urease redox center better than the hydrophilic chitosan. When chitosan was added without BSA (urease/chitosan/alginate/glutaraldehyde, cross-linking 3 in Table 1), the sensor sensitivity thus dropped by 60% as comparing the mixture with BSA (urease/chitosan/BSA/alginate/glutaraldehyde, cross-linking 2 in Table 1) (Figure 3A(5)).

The application of BSA and the cross-linking agent greatly improved the urea sensor temporal stability. The urea sensor with urease/BSA/alginate/glutaraldehyde (cross-linking 1 in Table 1) thus retained approximately 70% of its initial activity after 14 days (Figure 3B(3)). Sensors with entrapped urease/alginate (entrapped in Table 1) and urease/alginate/BSA (entrapped–stabilized in Table 1) lost more than 90 and 60%, respectively, of their activity after 7 days, most probably because of enzyme leaching (Figure 3B(1,2)). It was shown that BSA plays an important role as the urease stabilizer as sensors containing mixtures with and without BSA retained approximately 70 and 10%, respectively, of their sensitivity after 14 days of continuous use (Figure 3B(3,5)). A proposed schematic representation of the urease mode of action in the BSA/alginate mixture, cross-linked with glutaraldehyde and supplied with Ni^{2+}, is shown in Figure 4. First, the hexameric urease was supplied with its Ni^{2+} cofactors. Subsequently, BSA was added to stabilize the urea and to act as an amine group donor for the covalent cross-linking process with glutaraldehyde to the alginate polymer. The mixture was stabilized further by the addition of Ca^{2+} ions. In the presence of urea, the hexameric urease will dissociate to its active dimer form. Immobilization using BSA and cross-linking to alginate protect the active dimer from changing its conformation into inactive monomers.

Because of extensive potential application in medical or environmental monitoring and in the food industry, researchers have used a wide variety of techniques to achieve sensitive urea biosensors. The construction of urea biosensors are generally based on urease immobilized on electrode surfaces using many types of support such as cross-linking to nonconducting[46,47] or conducting[20,48] polymers or entrapment in nanostructures such as graphene.[18] Typically, entrapped urease is reported to have lost 90% if its activity after the first use/day because of leaching. Urease immobilized by cross-linking to nanostructures or polymers has been reported to retain 60–80% of its activity after several days and up to a week.[39] The decrease in urease activity could be due to leaching or inactivation of the enzyme. In our study, we showed that the addition of BSA as a stabilizing agent results in our sensors retaining approximately 70% sensitivity after 2 weeks.

Many of the earlier sensors focused on the detection of NH_4^+ formed as a hydrolysis product of urea. The reported sensor response times vary from 0.03 to 5 min (Table 2), depending on the electrode surface modification methods.

Urea detection through CO_2 as its hydrolysis product can be considered less attractive because of different speciations of CO_2 at different pH values. Suzuki et al.[26] constructed a urea

https://dx.doi.org/10.1021/acsomega.0c04146
ACS Omega 2020, 5, 27582–27590

biosensor based on urease in front of a Severinghaus-type CO_2 sensor. This sensor had a quite low limit of detection (LOD), however, bearing the disadvantages of Severinghaus sensors of slow response and unstable calibration curves, especially at low CO_2 concentrations.[28] The Severinghaus-based urea sensor required 12−30 min analysis time, depending on the urea concentration.[26]

The newly developed CO_2 microsensor has superior characteristics as compared to the Severinghaus-type sensors. Our CO_2 microsensor-based urea sensor using cross-linked urease/BSA/alginate/glutaraldehyde/Ca^{2+} in the front enzyme chamber exhibits a linear response to urea concentration from 0 to 1000 μM with a calculated LOD of 0.94 μM (3× blank SD/slope of calibration curve). It thus compares very positively with the previously reported urea biosensors (Table 2). Because of the electrochemistry being shielded by two gas-permeable membranes from possibly fouling dissolved organics, it can be used for continuous measurement without significant drift in the signal over a 24 h period. This is in strong contrast to detection with sensors without such membranes where there is a lack of records for continuous use over prolonged periods.

The need to remove inorganic carbon from the sample before analysis is the major disadvantage of urea analysis by this sensor. For use in continuous or semicontinuous monitoring, a CO_2 removal unit, thus, has to be implemented. As compared to NH_4^+ detection-based sensors, it is, however, relatively easy to remove CO_2 because of a 500-fold lower water solubility of CO_2 as compared to NH_3.

Sensor Reliability for Real Sample Analysis (Blood Plasma). The urea concentration in human blood plasma samples may vary from 1 to 10 mM depending on donor conditions. We are thus safely within the analytical range if the blood plasma is diluted by a factor of 10 before analysis. A spectrophotometric urea assay of the serum showed a concentration of approximately 2.4 ± 0.12 mM urea. The simple preparation of the blood plasma sample for biosensor analysis was as follows: the blood plasma was diluted 10 times with 10 mM citrate buffer pH 6 to convert most of the, at any moment, present carbonate species to CO_2 gas. Diluted plasma was then degassed with Ar/N_2 for 10 or 20 min. After 10 min, CO_2 was still detected at 8 μM concentration. Additional 10 min degassing removed the CO_2 to below the detection limit (Figure S5). After all the CO_2 was removed, the sample was analyzed using a calibrated urea sensor (0−1000 μM urea, R^2 = 0.999, Figure 5). The signal produced corresponded to a concentration of 247 ± 14 μM urea (sample n = 3, dilution factor 10×), corresponding to 97−106% of the 2.4 mM found by the spectroscopic assay on the undiluted sample.

CONCLUSIONS

A novel CO_2 microsensor-based urea microsensor has been developed. The urea biosensor has a linear range from 0 to 1000 μM with a calculated LOD of 0.94 μM and 95% response time of 120 s. The urea biosensor with urease in alginate, cross-linked with glutaraldehyde and Ca^{2+}, and with BSA as a stabilizer retained about 70% from its initial activity after 14 days. The urea biosensor was able to measure urea in blood plasma with a simple CO_2 removal pretreatment step. It would be preferable to have a sample pretreatment step to remove CO_2 integrated into the sensor, and this will be the focus of an upcoming study.

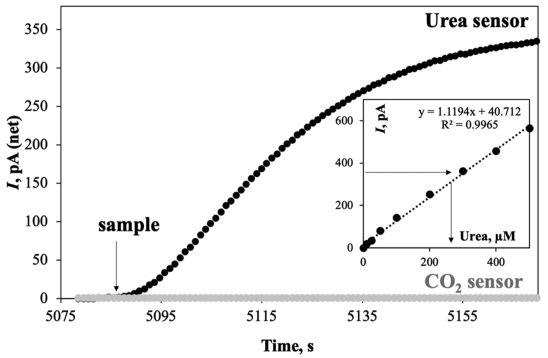


Figure 5. Urea biosensor signal after the addition of the blood plasma sample (diluted in buffer and CO_2-free). The gray symbols show the signal from a CO_2 microsensor that was inserted into the sample to verify the absence of CO_2 . The urea detection was completed after 100 s. Inset: urea sensor calibration, tip size 80 μm. Black arrows in the inserted graph: the signal from 10× diluted blood plasma indicating a urea concentration of 244 μM.

EXPERIMENTAL SECTION

Materials. Urease or urea amidohydrolase (EC 3.5.1.5) from Jack bean (*C. ensiformis*) with an activity of 100 U/mg solid was supplied from Toyobo Enzymes (Japan). This urease has an mw of 480 kDa with eight active sites and a pH stability between 5.5 and 8.5 with optimum pH at 6.[49] Alginic acid, low molecular weight chitosan (M_w: 50−190 kDa), BSA (66 kDa), glutaraldehyde, sodium citrate, $CaCl_2$, $NiSO_4$, $MnSO_4$, $CoCl_2$, $FeCl_3{\cdot}6H_2O$, H_2SO_4, diacetylmonoxime, thiosemicarbazide, PEGDMA, PVA-SBQ, *N*-(3-dimethylaminopropyl)-*N*′ ethyl-carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 1-ethyl-3-methylimidazolium dicyanamide (DCA) (≥98.0%), $CrCl_2$ (99.99%, beads), dimethylformamide (DMF), perfluoro methylcyclohexane, and Teflon AF 1600 were supplied from Sigma-Aldrich. Dow Corning (USA) supplied a Dow Corning 734 sealant.

Electrochemical Instrumentation. The current from the sensors was measured with a four-channel Unisense multimeter connected to a portable PC equipped with the program Sensor Trace Basic (Unisense A/S, Denmark). The multimeter can supply a polarization voltage from +2 to −2 V, and the polarization voltage could thus be adjusted to optimize sensor performance.

Colorimetric Instrumentation. The urea content and urease activity were both determined by colorimetric assays using the microplate reader Fluostar Omega with Omega data analysis software (BMG Labtech, Ortenberg, Germany).

CO_2 Microsensor. The idea was to create a urea biosensor by measuring the CO_2 formed by urease hydrolysis of urea. An amperometric CO_2 microsensor[29] was used as a transducer in the biosensor. The CO_2 microsensor contains a CO_2-reducing Ag cathode bathed in an ionic liquid (DCA) mixed with an aprotic solvent (DMF), a Ag guard cathode behind the sensing cathode to prevent interference from reducible contaminants in the ionic liquid, and a Ag pseudo-reference electrode. All the features are compartmentalized in glass casings, of which the front casing containing 1 M $CrCl_2$ in 0.1 M HCl served to remove otherwise interfering O_2. The general construction procedures have been described earlier[29,50] and will not be repeated here. The use of the aprotic solvent is dependent on application of a solvent-resistant membrane. The membrane in the inner Clark-type CO_2 sensor was made before assembly by

https://dx.doi.org/10.1021/acsomega.0c04146
ACS Omega 2020, 5, 27582−27590

briefly (0.5 s) dipping the tip region into 1% Teflon AF 1600 (0.01 g of Teflon AF 1600 in 1 mL of perfluoromethylcyclohexane), and waiting until the solvent evaporated (5−10 s), forming a nice membrane layer in the tip of the sensor with a thickness of 4−7 μm. After casting the Teflon AF membrane, the sensor tip was covered using a Dow Corning 734 silicone sealant so that a hybrid Teflon/silicone membrane was formed. The addition of the silicone layer ensured a good adherence of the membrane to the glass. The outer tip membrane was formed by insertion into uncured Dow Corning 734. The CO_2 microsensors used in this study had tip diameters of about 50 μm. The measuring and guard cathodes were polarized at −1.0 V against the internal Ag pseudo-reference electrode. It is actually now recommended to use a polarization of −1.1 V as this results in even better calibration curves for CO_2.[29]

CO_2 Calibration. The CO_2 calibrations were conducted by injection of small volumes of CO_2-saturated water (38 mM) into acidified (0.01 M HCl) demineralized water. The acidification ensured that all inorganic carbon was present as CO_2. Calibration points were read after sufficient times (1−2 min) to allow near complete sensor response. If not otherwise stated, the experiments were conducted at a room temperature of 20−22 °C.

Urease Immobilization and Integration with a CO_2 Microsensor to Form a Urea Biosensor. The CO_2 microsensor equipped with $CrCl_2$ trap was carefully inserted into a tapered glass chamber (enzyme chamber) with the 500 μm tip region containing the entrapped or cross-linked enzyme/alginate mixture, as will be described in detail later (Figure 1). The distance between the CO_2 sensor and enzyme chamber tips was about 200 μM, forming a biosensor with a 200 μm-long reaction chamber for urea hydrolysis by urease. Subsequently, the cross-linking of alginate was induced by inserting the biosensor tip into 100 mM $CaCl_2$ for at least 5 min. When glutaraldehyde was used as a cross-linker, the process was initiated by mixing the glutaraldehyde (0.5% final concentration) into the urease/alginate mixture. Immediately after mixing, the mixture was inserted into the urea sensor glass casing. To minimize enzyme leaching, the alginate-cross-linking process described above using $CaCl_2$ was also conducted. The CO_2 microsensor could now be inserted into the enzyme chamber and fastened in place with dental wax (Science Services GmbH, Germany), using an electrically heated NiChrome loop for local melting of the wax. It is important that there is a minimal gap between the CO_2 sensor tip and urease glass casing, as a poor fit will lead to slow 95% response. To maintain the alginate cross-linking and enzyme activity, the lumen behind the 500 μm-long alginate layer was filled with a solution containing 100 mM $CaCl_2$ and 10 mM $NiSO_4$. When the enzyme chamber needed to be replaced, the dental wax was melted to remove the old chamber, and a new chamber could be added (Figure 1). When the sensor was not in use, the sensor was stored and polarized at −1.0 V (vs Ag pseudo-reference) in degassed water containing 10 mM $CaCl_2$ and 1 mM $NiSO_4$.

Urea Calibration. Urea calibrations were conducted by injection of small volumes of 100 mM urea stock solution into degassed 10 mM citrate buffer solution at pH 6. The removal of CO_2 inside the biosensor by the CO_2 microsensor promotes conversion of bicarbonate to CO_2 in the strongly pH 6-buffered microenvironment. Calibration points were read after sufficient time (2−3 min) to allow near complete sensor response. If not otherwise stated, the experiments were conducted at a room temperature of 21 ± 1 °C.

Urea Concentration Determination in the Sample Using the Colorimetric Method. A blood plasma sample was obtained from a blood bank (Blodbanken, Aarhus University Hospital, Denmark). The blood plasma was taken from a pool of healthy patients. The urea concentration in the blood plasma was determined by a colorimetric urea assay.[8] Briefly, the urea assay uses urea solutions of 0, 10, 20, 30, 40, and 50 μM as the standard. The plasma sample was diluted with 10 mM citrate buffer pH 6 by a factor of 10−200 to find an optimum dilution factor. A 96 μL reagent 1 (55 μM $FeCl_3 \cdot 6H_2O$ in 6.6 M H_2SO_4 solution) was added to 420 μL sample/standard, followed by the addition of 30 μL of reagent 2 (323 mM diacetylmonoxime and 49 mM thiosemicarbazide in water). The mixture was incubated at 90 °C for 30 min and turned pink. The mixture was then transferred to a 96-well plate (Sarstedt AG&Co. KG, Germany), and the absorbance was measured at 520 nm using a microplate reader. The amount of urea in the plasma sample was calculated using a calibration plot from the standards (R^2 = 0.999).

Sample Treatment before Urea Measurement by the Urea Sensor. Blood plasma was diluted 10 times with 10 mM citrate buffer pH 6 to push the equilibrium between the inorganic C species toward CO_2. The diluted sample was then degassed with N_2 for 10−20 min, and the absence of CO_2 was verified with a CO_2 sensor. The CO_2 gas must be completely removed as it constitutes the only interference for our CO_2 microsensor-based urea determinations. After the CO_2 had been completely eliminated, the urea concentration could be measured using a calibrated urea sensor. The measurements of CO_2 and urea were performed in a chamber with flowing Ar/N_2 gas to prevent the diffusion of atmospheric CO_2 into the sample..

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c04146.

Urease activity measurement—Berthelot method CO_2 microsensor sensitivity during 5 months; effect of metal cofactor addition on urease activity; urea calibration with variable enzyme chamber lengths; effect of the urease immobilization method on urease activity; and CO_2 removal from serum by N_2 bubbling as observed with CO_2 microsensor (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Niels Peter Revsbech − *Aarhus University Centre for Water Technology (WATEC), Department of Biology, Aarhus University, Aarhus C 8000, Denmark;* orcid.org/0000-0002-2193-262X; Phone: +45 87156538; Email: revsbech@bios.au.dk

Authors

Deby Fapyane − *Aarhus University Centre for Water Technology (WATEC), Department of Biology, Aarhus University, Aarhus C 8000, Denmark*

Dmitriy Berillo − *Aarhus University Centre for Water Technology (WATEC), Department of Biology, Aarhus University, Aarhus C 8000, Denmark*

https://dx.doi.org/10.1021/acsomega.0c04146
ACS Omega 2020, 5, 27582−27590

Jean-Louis Marty − *BAE (Biocapteurs-Analyses-Environnement), Universite de Perpignan Via Domitia, Perpignan Cedex 66860, France*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c04146

Author Contributions

All authors have given approval to the final version of the manuscript and contributed equally. All authors contributed to design the experiments. D.F. and D.B. performed the experiments. D.F. analyzed the data and wrote the paper. D.B., J.-L.M., and N.P.R. revised the paper.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the Poul Due Jensen Foundation for its support to the sensor projects at Aarhus University Centre of Water Technology (WATEC). We thank Lars B. Pedersen and Mette Nikolajsen for their help in CO_2 microsensor production and testing.

■ REFERENCES

(1) Taylor, A. J.; Vadgama, P. Analytical reviews in clinical biochemistry: the estimation of urea. *Ann. Clin. Biochem.* **1992**, *29*, 245−264.
(2) Dimkpa, C. O.; Fugice, J.; Singh, U.; Lewis, T. D. Development of fertilizers for enhanced nitrogen use efficiency - Trends and perspectives. *Sci. Total Environ.* **2020**, *731*, 139113.
(3) Butzke, C. E.; Bisson, L. F. *Ethyl Carbamate Preventative Action Manual*; UC Davis Cooperative Extension, 1997.
(4) Jorgensen, N. O. G. Uptake of urea by estuarine bacteria. *Aquat. Microb. Ecol.* **2006**, *42*, 227−242.
(5) Recio, J.; Montoya, M.; Álvarez, J. M.; Vallejo, A. Inhibitor-coated enhanced-efficiency N fertilizers for mitigating NOx and N2O emissions in a high-temperature irrigated agroecosystem. *Agric. For. Meteorol.* **2020**, *292*−*293*, 108110.
(6) Francis, P. S.; Lewis, S. W.; Lim, K. F. Analytical methodology for the determination of urea: current practice and future trends. *TrAC, Trends Anal. Chem.* **2002**, *21*, 389−400.
(7) Koebel, M.; Elsener, M. Determination of urea and its thermal decomposition products by high-performance liquid chromatography. *J. Chromatogr. A* **1995**, *689*, 164−169.
(8) Coulombe, J. J.; Favreau, L. A new simple semimicro method for colorimetric determination of urea. *Clin. Chem.* **1963**, *9*, 102−108.
(9) Tran, T. Q. N.; Das, G. N.; Yoon, H. H.; Yoon, H. H. Nickel-metal organic framework/MWCNT composite electrode fornon-enzymatic urea detection. *Sens. Actuators, B* **2017**, *243*, 78−83.
(10) Bao, C.; Niu, Q.; Chen, Z.-A.; Cao, X.; Wang, H.; Lu, W. Ultrathin nickel-metal−organic framework nanobelt based electrochemical sensor for the determination of urea in human body fluids. *RSC Adv.* **2019**, *9*, 29474.
(11) Nguyen, N. S.; Yoon, H. H. Nickel oxide-deposited cellulose/CNT composite electrode for non-enzymatic urea detection. *Sens. Actuators, B* **2016**, *236*, 304−310.
(12) Madhura, T. R.; Kumar, G. G.; Ramaraj, R. Reduced graphene oxide supported 2D-NiO nanosheets modified electrode for urea detection. *J. Solid State Electrochem.* **2020**, DOI: 10.1007/s10008-020-04763-3.
(13) Zhang, Y.; Liu, Y.-Q.; Bai, Y.; Chu, W.; Sh, J. Confinement preparation of hierarchical NiO-N-doped carbon@reduced graphene oxide microspheres for high-performance non-enzymatic detection of glucose. *Sens. Actuators, B* **2020**, *309*, 127779.
(14) Andrews, R. K.; Dexter, A.; Blakeley, R. L.; Zerner, B. Jack bean urease (EC 3.5. 1.5) VIII. On the inhibition of urease by amides and esters of phosphoric acid. *J. Am. Chem. Soc.* **1986**, *108*, 7124−7125.
(15) Krajewska, B. Ureases I. Functional, catalytic and kinetic properties: A review. *J. Mol. Catal. B: Enzym.* **2009**, *59*, 9−21.
(16) Barrios, A. M.; Lippard, S. J. Interaction of urea with a hydroxide-bridged dinuclear nickel center: an alternative model for the mechanism of urease. *J. Am. Chem. Soc.* **2000**, *122*, 9172−9177.
(17) Bertocchi, P.; Compagnone, D.; Palleschi, G. Amperometric ammonium ion and urea determination with enzyme-based probes. *Biosens. Bioelectron.* **1996**, *11*, 1−10.
(18) Srivastava, R. K.; Srivastava, S.; Narayanan, T. N.; Mahlotra, B. D.; Vajtai, R.; Ajayan, P. M.; Srivastava, A. Functionalized multilayered graphene platform for urea sensor. *ACS Nano* **2011**, *6*, 168−175.
(19) Cho, W.-J.; Huang, H.-J. An amperometric urea biosensor based on a polyaniline− perfluorosulfonated ionomer composite electrode. *Anal. Chem.* **1998**, *70*, 3946−3951.
(20) Hao, W.; Das, G.; Yoon, H. H. Fabrication of an amperometric urea biosensor using urease and metal catalysts immobilized by a polyion complex. *J. Electroanal. Chem.* **2015**, *747*, 143−148.
(21) Vardar, G.; Altikatoglu, M.; Isildak, I. An all solid-state urea biosensor based on ammonium-selective PVC membrane electrode. *FEBS J.* **2016**, *283*, 272−273.
(22) Kumar, V.; Kaur, I.; Arora, S.; Mehla, R.; Vellingiri, K.; Kim, K.-H. Graphene nanoplatelet/graphitized nanodiamond-based nanocomposite for mediator-free electrochemical sensing of urea. *Food Chem.* **2020**, *303*, 125375.
(23) Park, M.; Kim, J. Y.; Kim, K.; Pyun, J.-C.; Sung, G. Y. Parylene-Coated Polytetrafluoroethylene-Membrane-Based Portable Urea Sensor for Real-Time Monitoring of Urea in Peritoneal Dialysate. *Sensors* **2019**, *19*, 4560.
(24) Guilbault, G. G.; Shu, F. R. Enzyme electrodes based on the use of a carbon dioxide sensor: Urea and L-tyrosine electrodes. *Anal. Chem.* **1972**, *44*, 2161−2166.
(25) Ruzicka, J.; Hansen, E. H.; Ghose, A. K.; Mottola, H. A. Enzymic determination of urea in serum based on pH measurement with the flow injection method. *Anal. Chem.* **1979**, *51*, 199−203.
(26) Suzuki, H.; Arakawa, H.; Karube, I. Performance characteristics of a urea microsensor employing a micromachined carbon dioxide electrode. *Electroanalysis* **2000**, *12*, 1327−1333.
(27) Severinghaus, J. W.; Bradley, A. F. Electrodes for blood pO2 and pCO2 determination. *J. Appl. Physiol.* **1958**, *13*, 515−520.
(28) Xie, X.; Bakker, E. Non-Severinghaus potentiometric dissolved CO2 sensor with improved characteristics. *Anal. Chem.* **2013**, *85*, 1332−1336.
(29) Fapyane, D.; Revsbech, N. P. Fast Responding Amperometric CO2 Microsensor with Ionic Liquid-Aprotic Solvent Electrolyte. *ACS Sens.* **2020**, *5*, 2604−2610.
(30) Larsen, L. H.; Revsbech, N. P.; Binnerup, S. J. A microsensor for nitrate based on immobilized denitrifying bacteria. *Appl. Environ. Microbiol.* **1996**, *62*, 1248−1251.
(31) Nielsen, M.; Larsen, L. H.; Jetten, M. S. M.; Revsbech, N. P. Bacterium-based NO2− biosensor for environmental applications. *Appl. Environ. Microbiol.* **2004**, *70*, 6551−6558.
(32) Fearon, W. R. Urease. Part I. The chemical changes involved in the zymolysis of urea. *Biochem. J.* **1923**, *17*, 84−93.
(33) Balasubramanian, A.; Ponnuraj, K. Crystal structure of the first plant urease from jack bean: 83 years of journey from its first crystal to molecular structure. *J. Mol. Biol.* **2010**, *400*, 274−283.
(34) Zambelli, B.; Musiani, F.; Benini, S.; Ciurli, S. Chemistry of Ni2+ in urease: sensing, trafficking, and catalysis. *Acc. Chem. Res.* **2011**, *44*, 520−530.
(35) Carter, E. L.; Flugga, N.; Boer, J. L.; Mulrooney, S. B.; Hausinger, R. P. Interplay of metal ions and urease. *Metallomics* **2009**, *1*, 207−221.
(36) Hirai, m.; Kawai-hirai, R.; Hirai, T.; Ueki, T. Structural change of jack bean urease induced by addition surfactants studied with synchrotron-radiation small-angle X-ray scattering. *Eur. J. Biochem.* **1993**, *215*, 55−61.

(37) Sehgal, P. P.; Naylor, A. W. Purification and Properties of Urease Derived from Hydrated Seeds of Jack Bean, Canavalia ensiformis (L) DC. *Plant Physiol.* **1966**, *41*, 567−572.
(38) Omar, S.; Beauregard, M. Dissociation and unfolding of jack bean urease studied by fluorescence emission spectroscopy. *J. Biotechnol.* **1995**, *39*, 221−228.
(39) Krajewska, B. Ureases. II Properties and their customizing by enzyme immobilizations: A review. *J. Mol. Catal. B: Enzym.* **2009**, *59*, 22−40.
(40) Jasti, L. S.; Dola, S. R.; Kumaraguru, T.; Bajja, S.; Fadnavis, N. W.; Addepally, U.; Rajdeo, K.; Ponrathnam, S.; Deokar, S. Protein-coated polymer as a matrix for enzyme immobilization: Immobilization of trypsin on bovine serum albumin-coated allyl glycidyl ether−ethylene glycol dimethacrylate copolymer. *Biotechnol. Prog.* **2014**, *30*, 317−323.
(41) Kumar, S.; Dwevedi, A.; Kayastha, A. M. Immobilization of soybean (Glycine max) urease on alginate and chitosan beads showing improved stability: Analytical applications. *J. Mol. Catal. B: Enzym.* **2009**, *58*, 138−145.
(42) Doğaç, Y. I.; Teke, M. Synthesis and characterisation of biocompatible polymer-conjugated magnetic beads for enhancement stability of urease. *Appl. Biochem. Biotechnol.* **2016**, *179*, 94−110.
(43) Turbett, G. R.; Høj, P. B.; Horne, R.; Mee, B. J. Purification and characterization of the urease enzymes of Helicobacter species from humans and animals. *Infect. Immun.* **1992**, *60*, 5259−5266.
(44) Simpliciano, C.; Clark, L.; Asi, B.; Chu, N.; Mercado, M.; Diaz, S.; Goedert, M.; Mobed-Miremadi, M. Cross-linked alginate film pore size determination using atomic force microscopy and validation using diffusivity determinations. *J. Surf. Eng. Mater. Adv. Technol.* **2013**, *03*, 1−12.
(45) Phan, H. T. M.; Bartelt-Hunt, S.; Rodenhausen, K. B.; Schubert, M.; Bartz, J. C. Investigation of bovine serum albumin (BSA) attachment onto self-assembled monolayers (SAMs) using combinatorial quartz crystal microbalance with dissipation (QCM-D) and spectroscopic ellipsometry (SE). *PLoS One* **2015**, *10*, No. e0141282.
(46) Mascini, M.; Guilbault, G. G. Urease coupled ammonia electrode for urea determination in blood serum. *Anal. Chem.* **1977**, *49*, 795−798.
(47) Razumiene, J.; Sakinyte, I.; Kochane, T.; Maciulyte, S.; Straksys, A.; Budriene, S.; Barkauskas, J. Carbon Electrode based Urea Sensor. *Proceedings of the International Conference on Biomedical Electronics and Devices* (BIODEVICES); SciTePress, 2013; pp 197−201.
(48) Luo, Y.-C.; Do, J.-S. Urea biosensor based on PANi(urease)-Nafion/Au composite electrode. *Biosens. Bioelectron.* **2004**, *20*, 15−23.
(49) Gorin, G.; Chin, C.-C. Urease IV. Its reaction with N-ethylmaleimide and with silver ion. *Biochim. Biophys. Acta, Enzymol. Biol. Oxid.* **1965**, *99*, 418−426.
(50) Revsbech, N. P.; Garcia-Robledo, E.; Sveegaard, S.; Andersen, M. H.; Gothelf, K. V.; Larsen, L. H. Amperometric microsensor for measurement og gaseous and dissolved CO2. *Sens. Actuators, B* **2019**, *283*, 349−354.

https://dx.doi.org/10.1021/acsomega.0c04146
ACS Omega 2020, 5, 27582−27590