For control of diabetes mellitus, maintenance of blood glucose concentrations within the normal physiological range is critical to minimizing diabetic complications. As a result, accurate in vivo and in vitro measurement of glucose concentration in physiological fluids has long been a central goal of biosensor research. Traditionally, glucose biosensors employ indirect detection of the products of the glucose oxidase or glucose dehydrogenase reactions, although direct detection has also been reported. In general, glucose oxidase has been the favored biological recognition element due to the high binding specificity, high turnover rate, and high stability of this enzyme. However, indirect detection of hydrogen peroxide, or other products, often suffers from low selectivity arising from chemical interference by other easily reducible substances, such as ascorbate and urate. Direct detection suffers from two general drawbacks that limit the efficiency with which electrons can be collected: proteins are usually electrically insulating, and the reaction site may be located far from the collection electrode. In addition, electron transfer proteins are also required. To circumvent these problems, the current report focuses on direct glucose detection by electrochemical impedance using the d-glucose/galactose receptor (GGR) from E. coli.

The potential use of the bacterial periplasmic binding protein superfamily (bBPP) in biosensors has arisen due to their solubility, stability and ability to reversibly bind a large variety of small ligands including sugars, amino acids, and inorganic ions. These proteins comprise a large family of functionally similar receptors with a two-domain structure that exhibit a large hinge-twist conformational change upon ligand binding. Whereas the proteins are open and flexible when the ligand is absent, they are more structurally compact and closed when the ligand is bound. This large amplitude motion can be exploited for biosensor development of small molecules.

The GGR protein is specific for glucose or galactose and binds these ligands in the micromolar range, which is more sensitive than that needed for blood glucose. A number of groups have detected sugars by labeling this protein with fluorescent tags in sites where the chemical environment is altered during ligand binding. Recent studies have used surface plasmon resonance (SPR) methods with this protein for glucose detection. Our methodology uses the GGR protein without labels and employs simpler and more general electrochemical impedance measurements to probe glucose binding via this conformational change. The GGR protein, shown in Fig. 1, is immobilized onto an Au surface through formation of an Au-S bond to a genetically engineered cysteine residue at the N-terminus of the protein. We clearly show that this methodology of reagentless glucose detection yields reproducible glucose-specific signals and has potential for direct continuous monitoring of glucose.

Manuscript submitted November 22, 2004; revised manuscript received April 12, 2005. Available electronically June 7, 2005.

Experimental

Expression and purification of GGR A1C.—The GGR A1C plasmid was expressed in E. coli BL21 cells and purified as described previously. Protein was then dialyzed against two exchanges of 3 M guanidinium chloride (GnHCl), 100 mM KCl, 20 mM EDTA, 10 mM Tris pH 7.1, and four exchanges of refolding buffer containing 100 mM KCl, 10 mM Tris pH 7.1, 0.5 mM CaCl₂. Quantitation of the proteins was determined by A₂₈₀. Figure 1 illustrates the three-dimensional structure of the GGR A1C, including the placement of the cysteine group to the N-terminus of the protein.

Electrode preparation.—Glass slides with a 100 nm Au film atop a 5 nm Ti adhesion layer were purchased from Evaporated Metal Film Corporation (Ithaca, NY). The electrode was fixed by an O-ring into an electrochemical cell constructed from virgin Teflon. To minimize the amount of protein needed, the conical electrochemical cell was designed with an electrode area of 0.28 cm² and a cell volume of 5 mL. The electrode was cleaned electrochemically by potential scanning in a solution containing 0.1 M H₂SO₄ and 0.01 M KCl. The Au electrode was exposed to 50 μL of 27 μM GGR A1C, then the cell was incubated for 3-5 h. Then Au electrodes were subsequently thoroughly rinsed with 10 mM PH 7.1 tris buffer with 0.1 M KCl and 0.5 mM CaCl₂, and then immersed into the buffer solution.

Instruments.—Impedance measurements were obtained with a standard three-electrode electrochemical cell, with a Pt counter electrode and a saturated calomel (SCE) reference electrode. Impedance data was obtained with two different experimental setups, one for steady state and another for transient measurements. The complete frequency spectrum from 10⁻² to 10³ Hz can be measured at steady state with a Bioanalytical Systems (BAS) model 100B/W electrochemical workstation, with a built-in multi-sine ac impedance module. Temporal evolution of the impedance at a single frequency was measured with a Princeton Applied Research (PAR) model 263A potentiostat and a Stanford Research Systems SR830 lock-in amplifier, for phase sensitive detection.

Experimental methods.—The electrode was first immersed into the test solution (pH 7.1 10 mM Tris buffer with 0.1 M KCl and 0.5 mM CaCl₂). The open-circuit potential (OCP), where the net current is zero, was measured, and the dc potential during subsequent measurements was set to this value. For different experiments, the OCP varied from 85-95 mV SCE when no redox probe is included in the analyte. After measuring the impedance of the biosensor interface, glucose or fructose in the same refolding buffer was added to the cell up to the desired final ligand concentration. The ligand solution was added at a position ~2 cm above the electrode surface, so many of the measurements reported below correspond to a diffusion-limited biosensor. For the steady-state experiments, the impedance
of the biosensor interface was measured periodically until reaching steady state. For the transient experiments, the impedance was recorded at 15 min intervals.

Results and Discussion

In most impedance biosensor studies, a redox probe such as \( \text{Fe(CN)}_{6}^{3-/4-} \) is added to the analyte to decrease the measured impedance value. Otherwise, the ac current response that is measured becomes quite small, which may decrease the experimental sensitivity. Biological recognition is then detected as a change in the electron transfer kinetics of the redox probe, or as a change in the interfacial capacitance. For the Au-immobilized D-glucose/galactose receptor protein studied here, the addition of a \( \text{Fe(CN)}_{6}^{3-/4-} \) redox probe adversely impacts the stability of the impedance signal, as shown in Fig. 2 as a Nyquist representation for frequencies from 1.2 to 950 Hz. Here the redox probe concentrations are 0.5 mM \( \text{K}_{4}\text{Fe(CN)}_{6} + 0.5 \text{mM K}_{3}\text{Fe(CN)}_{4} \), and the dc potential is the OCP for this electrochemical system (+186 mV SCE).

In a Nyquist representation, the real component (in-phase) of the complex impedance is shown on the abscissa, and the imaginary component (out-of-phase) on the ordinate. For simple electrical circuits, a resistive impedance is real and a capacitive impedance imaginary. In Fig. 2, the lowest frequency results correspond to the highest values of the real impedance, the highest frequency results to the lowest real impedance values. The approximately semicircular plot in Fig. 2 is typically seen in simple electrochemical systems. The diameter of the semicircle corresponds to the charge transfer resistance, which is inversely related to the charge transfer rate at the interface. The gradual increase in the charge transfer resistance seen here is consistent with gradual protein denaturation, which increases the extent to which the Au surface is obscured by the protein. Results at other dc potentials, including the potential employed below when the redox probe is omitted, were also unstable.

The results shown in Fig. 2 are consistent with the use of \( \text{Fe(CN)}_{6}^{3-} \) as a mild oxidant that can denature some proteins. However, other reports have suggested that \( \text{Fe(CN)}_{6}^{3-/4-} \) interactions with proteins are either more complex or in some cases benign. \( \text{Fe(CN)}_{6}^{3-/4-} \) has also been reported to disrupt the formation of Au-thiol self-assembled monolayers (SAM) through mild etching of Au. Although the \( \text{Fe(CN)}_{6}^{3-} \) complex is quite stable, a small concentration of free \( \text{CN}^- \) will always be present.

Figure 3 shows the Nyquist representation of the impedance results from 1.2 to 950 Hz for the GGR/Au biosensor interface first in a blank buffer solution, then following addition of glucose. In both cases data is obtained only after reaching steady state. All of the data shown in Fig. 3 were obtained in the absence of \( \text{Fe(CN)}_{6}^{3-/4-} \). The imaginary component of the impedance is consistently lower upon introduction of glucose. Note that the magnitude of the impedance measured is quite large, and the Nyquist plot is close to a vertical line, often indicating a capacitive interface. However, in the present case, this instead reflects the omission of a redox probe, which is necessary to obtain stable impedance values. Thus, no electrochemically active reagents have been added to the analyte. If fructose, a nonbinding sugar, is added to the blank buffer used in Fig. 3, no change in the impedance signal is observed, demonstrating that the biosensor response is specific to glucose.

In repeat experiments, the magnitude of the impedance at a frequency of 20 Hz is consistently reduced by about 3-8% upon glu-
The measured impedance, with and without glucose introduction, is quite stable. However, for subsequently prepared GGRAIC/Au biosensor interfaces, the magnitude of the impedance can vary by \( \sim 20\% \). Although the interface is clearly capacitive, the results shown in Fig. 3 do not fit simple equivalent circuit models based on a Randles model, even with inclusion of a Warburg impedance. Complete understanding of these results in terms of equivalent circuit models will be the subject of future investigations.

Once steady state is reached, acquisition of the entire impedance spectrum shown in Fig. 3 takes \( \sim 5 \) min. A faster response may be needed in some biosensor applications, so the impedance at one frequency can be monitored instead. Figure 4 shows the time evolution of the magnitude of the impedance at 20 Hz, before and after introduction of glucose to a final concentration of 10 \( \mu \)M. In this system, steady state is not reached for \( \sim 130 \) min due to the method for introducing glucose, which takes significant time to diffuse to the surface. Thus, Fig. 4 illustrates the concept of monitoring the impedance at one frequency, but does not produce a rapid response. A faster response requires the use of a flow cell or other means of agitation, such as sonication. The incubation time for the GOR protein to bind its natural ligands is quite rapid, as expected given its role in chemotaxis, so a much more rapid response can be obtained with an improved cell design.

For many applications, a linear biosensor response is desired in order to obtain quantitative analyte concentrations. Further experiments were conducted with glucose concentrations in the \( \mu \)M range, because linearity will most likely be observed near the equilibrium dissociation constant of this protein, \( \sim 0.2 \) \( \mu \)M.\(^{26}\) Figure 5 illustrates the response of the current impedance biosensor as a function of glucose concentration, choosing as the response the change in the impedance magnitude after 30 min, illustrating that a linear response can be obtained. Note that physiological glucose concentrations typically range from 1-30 mM. The much lower concentration range in the current report may be reached simply by dilution. Alternatively, the equilibrium dissociation constant can be dramatically increased by introducing residues near the ligand binding pocket that reduce this protein’s affinity for glucose.\(^{12}\) This should allow the equilibrium dissociation constant to be increased into the physiological range, although multiple substitutions will likely be required.\(^{12}\)

The application of electrochemical impedance methods to probing biomolecular interactions at surfaces has been recently reviewed.\(^{35}\) Electrochemical impedance has been previously demonstrated as the transduction method for biological recognition of protein antigens, DNA, and bacteria. Even so, in many cases, impedance detection requires conjugation with nanoparticles or protein molecules, metalization, or indirect detection through formation of an insoluble product.\(^{26-29}\) In these systems, large species are either introduced or accumulated so that the biosensor interface is significantly perturbed. A reagentless biosensor system, where no “tagging” is needed would clearly be preferable.

A general electrochemical impedance method for the reagentless, direct detection of small molecule ligands or substrates such as glucose that are often of interest for biomedical applications has not been demonstrated. This direct detection is far more challenging, as the biosensor interface is normally only slightly perturbed. The critical elements of our methodology are the use of “hinge-bending” proteins and the use of electrochemical methods to directly sense the large amplitude motion that occurs during biological recognition. Electrochemical impedance methods should be capable of sensing ligand binding events that bury hydrophilic residues and ligand binding events that change the total protein surface area due to large amplitude motion. In addition, an important advantage of impedance-based biosensors is that they are not restricted to redox enzymes, greatly increasing the number of proteins that can be employed for biomolecular recognition.

Acknowledgments

This work has been supported by NSF (CTS-0329698) and NIH (RO3-CA 89705).

Clarkson University assisted in meeting the publication costs of this article.

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