Enzyme-Based Biosensors for in Vivo Measurements

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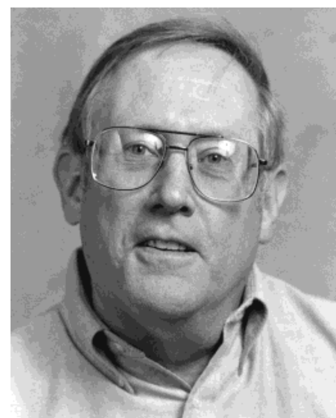
I. Introduction

The genesis of the biosensor undoubtedly began with the efforts of Leland C. Clark, Jr. to measure oxygen in biological fluids.[1] The key innovation was the placement of *both* the indicating and reference electrodes behind a gas-permeable membrane, thus isolating them from the biological fluid. The concept of an enzyme-based device was presented by Clark in 1962 at a New York Academy of Sciences Symposium[2] and was realized as a commercial clinical analyzer (Yellow Springs Instruments) in 1975. Clark has given an interesting personal account of his work in this area.[3] Significant progress has been made in the development of sensors capable of in vivo measurements, the most demanding of applications, and this will be the focus of the present review. The results are a consequence of proliferating cross-disciplinary interactions involving electrochemistry, spectroscopy, materials science, bioengineering, and medicine.

This review will focus on significant advances in in vivo biosensing, most of which has occurred in the last 10 years. The literature for biosensors is vast, but the number of sensors with demonstrated capabilities for in vivo operation is quite limited. Some applications in cell cultures are cited, but these have in common with in vivo sensors the requirement that the sensor is “reagentless”, i.e., no reagents, which might otherwise perturb the system, need be added. A further requirement is that the sensor must be capable of continuous measurements at least over a period of several hours.

Because enzyme-based sensors tend to either produce or consume protons and/or electroactive species, the vast majority of enzyme sensors use an electrode as the transducer, which will be the primary emphasis of this review. From a practical and commercial point of view, only four sensors have been widely used: glucose (diagnosis and treatment of diabetes, food science, biotechnology), lactate (sports medicine, critical care, food science, biotechnology), urea (clinical applications), and glutamate/glutamine (food science, biotechnology). However, glucose is, by far, the most widely employed and therefore continues to drive research toward better sensors. Devices capable



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of in vivo monitoring of blood glucose as part of an "artificial pancreas" have been the subject of extensive research by many groups over the last 15 years.[4-19]

Most enzyme-based biosensors employ a class of enzymes known as oxidoreductases, and the two most frequently encountered subclasses are the oxidases and dehydrogenases. Their reaction sequences can be described by the following reactions:

$$S_{red} + E_{ox} \rightarrow S_{ox} + E_{red} \quad (1)$$

$$S'_{ox} + E_{red} \rightarrow S'_{red} + E_{ox} \quad (2)$$

If, for example, glucose oxidase (GOx) is the enzyme employed, then S_{red}, S_{ox}, S'_{ox}, and S'_{red} correspond to glucose, gluconic acid, oxygen, and hydrogen peroxide, respectively. To make reaction eq 1 rate limiting, a large excess of S'_{ox}, typically oxygen, is required. This is accomplished by increasing the flux ratio of S_{red} to S'_{ox} into the enzyme reaction layer such that S'_{ox} is in large excess. When properly implemented, the sensor output is largely independent of oxygen partial pressure over a wide range down to 8 Torr. (Subcutaneous tisssue levels of oxygen are estimated at 20–30 Torr.)[20]

The rate of the overall reaction sequence can be measured by monitoring the consumption of S'_{ox} or the formation of S'_{red}. In practical GOx systems this means either the consumption of oxygen or the production of peroxide. The advantage of the former approach is the relative ease of separation (as Clark demonstrated) of oxygen from other electroactive species through the use of a gas-permeable membrane. The disadvantage of this approach is that it is more complicated, requiring two measurements (concentration of oxygen in the presence and absence of the enzymatic reaction). However, the approach is capable of compensating for significant fluctuation in oxygen levels.[21] Measurement of peroxide formation has the advantage of being simpler, especially for small sensors, however, with the disadvantage that a number of in vivo endogenous species (ascorbate and urate) are electroactive at the applied potential required for peroxide oxidation. It has proven possible to use permselective membranes that can successfully exclude interfering species.[19] Monitoring of peroxide reduction is not possible because of the inability to find a potential at which peroxide, but not oxygen, is reduced. A porous Teflon membrane has been used to electrochemically detect hydrogen peroxide because of its "gaslike" properties,[22] thus providing selectivity.

It is obvious then that two redox couples are necessary to carry out the enzyme-catalyzed redox reaction, one of which is the analyte. Thus, the other (S'_{ox}) has to be introduced in some fashion. It is often desirable for the biosensor to be "reagentless". This means that a clever method for delivering the second substrate (cofactor) must be devised or S'_{ox} must initially be present at sufficiently elevated levels that it does not affect sensor response. This is the reason for the overwhelming preference for oxidases, because oxygen is often naturally already present at the required levels. If the enzyme belongs to the dehydrogenase class, it will typically use NAD as a cofactor. Either NAD or NADH will have to be monitored electrochemically or by some other means. There are a large number of enzymes in this class, but the need to add reagent (NAD) and the relative complexity of NADH electrochemistry have precluded their use in in vivo applications.

Alternatively, S'_{ox} could be eliminated if the redox center of the enzyme was coupled *directly* to the electrode, thus making the electrode the "sink" for the electrons needed to complete eq 2. With a few exceptions, this has not proven to be feasible because enzyme redox centers are frequently well buried within the protein and heterogeneous electron transfer is prohibitively slow. Horseradish peroxidase, which is a very small enzyme, is able to communicate directly with the electrode in the case of carefully prepared carbon electrodes[23] or through the use of 30 nm gold particles deposited on a gold electrode.[24]

II. Mediated Electron Transfer

If direct electron transfer between the electrode and the redox center of the enzyme is not possible, then mediators must be employed; however, they must be prevented from diffusing out of the enzyme layer and into the biological medium. The pioneering work of Heller[25] demonstrated that it was possible to derivatize GOx with ferrocene, thus promoting relayed electron transfer to an electrode. A detailed experimental and theoretical study by Mikkelsen and co-workers[26] revealed that the rates of intramolecular electron transfer are very sensitive to the particular ferrocene derivative employed and also to the distance between the flavin ring and the point of covalent attachment of the mediator. It is not necessary to have a large number of mediator molecules attached; they need instead to be in the "right" locations. The highest rate of intramolecular electron transfer was obtained for a ferrocene carboxylic acid derivative (0.9 s^{-1}). If the rate of reaction of oxygen with the enzyme is assumed to be 2×10^6 M^{-1} s^{-1}, then the "relay rate" would have to be 5×10^3 s^{-1} if the sensor is operating in air saturated solution ($[O_2]$ = 240 μM). Faster relay rates were obtained using dopamine and daunomycin[27] compared to the ferrocene derivatives, and targeting carboxylate attachment points rather than amine functions, because the former are significantly closer to the flavin ring, also helped to improve efficiency. Although the resulting relay rates were significantly faster, they were still several orders of magnitude short of what would be required. This illustrates a fundamental dilemma: the mediator must be able to compete with freely diffusing oxygen, which can have a parasitic effect on sensor response, and this is quite difficult if it must be anchored in place. It was observed that sensors based on hydrogels made by cross-linking GOx and the redox polymer formed from complexing poly(vinyl pyridine) with $[Os(4,4'\text{-dimethoxy-}2,2'\text{-bipyridine})_2Cl]^{+/2+}$ showed oxygen effects (signal decrease) of between 2% and 41%, depending on the glucose concentration.[28] The low potential (+35 mV vs SCE) reduces significantly but does not eliminate ascorbate effects. Heller's group demonstrated that

oxygen interference can be largely reduced by "salting out" oxygen so that it does not enter the reaction layer.[29] Willner and co-workers elegantly addressed the question of whether it is possible to "wire" an enzyme to an electrode with sufficiently high efficiency to prevent competition from oxygen.[30] The strategy was to remove the noncovalently bound FAD from the enzyme (GOx) and attach it to a tether consisting of cystamine chemisorbed on a gold electrode surface and a pyrroloquinoline quinone (PQQ) link. The transfer of electrons from the enzyme to the electrode yielded an apparent turnover number (maximum rate for the enzymatic reaction) of 900 ± 150 s^{-1} compared to the value for the enzyme in solution of about 1000 s^{-1}, indicating excellent coupling efficiency. There is only a monolayer of enzyme on the electrode surface, and even assuming relatively high stability for GOx, it is unclear whether such a sensor would have a reasonable lifetime. This work, however, emphasizes that careful control of the communication link between the electrode and redox center of the enzyme is extremely important.

Many oxidases contain a flavin moiety as the redox center, and the potential of this functionality is sufficiently low that mediators having potentials in the range from −100 to +35 mV vs AgCl/Ag reference can be employed. In this range the formal potentials of mediators are high enough to rapidly facilitate oxidation of the enzyme but low enough to largely avoid either direct or mediated oxidation of endogenous reducing agents such as ascorbate. Depending on the particular application, the mobility of the mediator may also be an issue. For example, electrochemically based glucose self-monitoring ("fingerstick") systems use mediators such as ferricyanide or ferrocene derivatives that are free to diffuse out of the enzyme layer.[31] This is entirely adequate for measurements of 30 s duration but not for applications demanding sensor stability for days, weeks, or months.

In addition to ferrocene derivatives including those attached to polymers,[32] other mediators have also been investigated. Nickelocene has a lower formal potential than ferrocene[33] but also reacts more slowly with the enzyme.[34] Phenazines, phenoxazines, and Wurster's salts have been evaluated as possible mediators.[34] In addition to the redox hydrogels developed by Heller's group and others, sensors have been prepared using polymers, to which mediators have been attached,[35] and deposited on an electrode. An intriguing system yielding surprisingly stable sensors is constructed from an electrode coated with the organic salt tetrathiafulvalene (TTF) tetracyanoquinodimethane (TCNQ). First described by Kulys[36] in the 1980s, this electrode material appears to mediate electron transfer to the enzyme by corroding, yielding primarily TTF^+, which then functions as the electron acceptor for reaction 2.[37] Reasonable apparent stability can be achieved, but because TTF^+ is also not stable,[38] it is likely that the observed performance is attributable to constant renewal of the mediator. Khan[39] has investigated in some detail the morphology of the organic salt surface as this has a significant effect on sensor stability and sensitivity. A high rate of mediator efficiency, as measured by apparent Michaelis constants for glucose in the range of 20−60 mM, was achieved by preparing dendritic or "tree-like" deposits. The sensors showed very little influence of oxygen and interferents such as ascorbate. Mediators have been "stored" in carbon paste so that they may freely diffuse. This is frequently a good solution provided that the diffusion of the mediator into the test medium does not create any problems. A recent such example is the creation of an oxygen reservoir.[40] Most investigations have reached the same conclusion: it is extremely difficult to find mediators that react rapidly with the redox center of the enzyme, thus avoiding oxygen interference, and can also survive repeated recycling without degradation or leaching, especially when placed in a biological milieu. Proper sensor design can tolerate some loss of enzyme activity during use without affecting sensor performance. Much less tolerance is permissible in the case of the mediator unless a constant and renewable supply is available.

The appeal of mediator-based sensors for in vivo measurements is the expectation that their response might, in principle, be independent of oxygen tension. This is particularly important for lactate or glutamate monitoring under conditions where oxygen levels are expected to be low (ischemia). Perhaps one of the earliest examples is that of Boutelle and co-workers,[41] who employed the organic salt, TTF^+TCNQ^-, as the mediator in the measurement of brain glucose with a 250 μm sensor. They report 31% activity remaining after 28 days of implantation. It has been demonstrated[37] that TTF^+, released from the organic salt coating in a soluble form, serves as the mediator. Reaction of the mediator with endogenous reductants such as ascorbate must be taken into account. Heller's group has employed redox hydrogels for sensors implanted in the vascular bed[42] and subcutaneous tissue of rats.[43] Other examples of mediator-based sensors are given subsequently.

III. Practical Considerations for in Vivo Sensors

There are a number of routes to monitoring of biological systems including batch sampling of biological fluids (blood, urine, etc.), indwelling catheters, microdialysis, and some noninvasive monitoring techniques such as pulse oximetry and imaging, however, few can match the temporal and spatial resolution possible with microsensors. Unfortunately, the requirements for a successful biosensor are sufficiently strict that most sensors cannot meet them and thus remain mostly laboratory curiosities. These include sensitivity, selectivity, detection limit, stability, and response time. Since enzyme-based sensors must consume analyte in order to measure it, high sensitivity may correlate with high analyte consumption and can result in depletion effects in the vicinity of the sensor. This is a particularly serious problem in tissues of high metabolic activity. Selectivity, as we will see subsequently, is evaluated in vitro in two different ways: as a ratio of currents for the analyte and the interferent at the same concentration or as a signal response when several species are present simultaneously. This latter approach allows for the

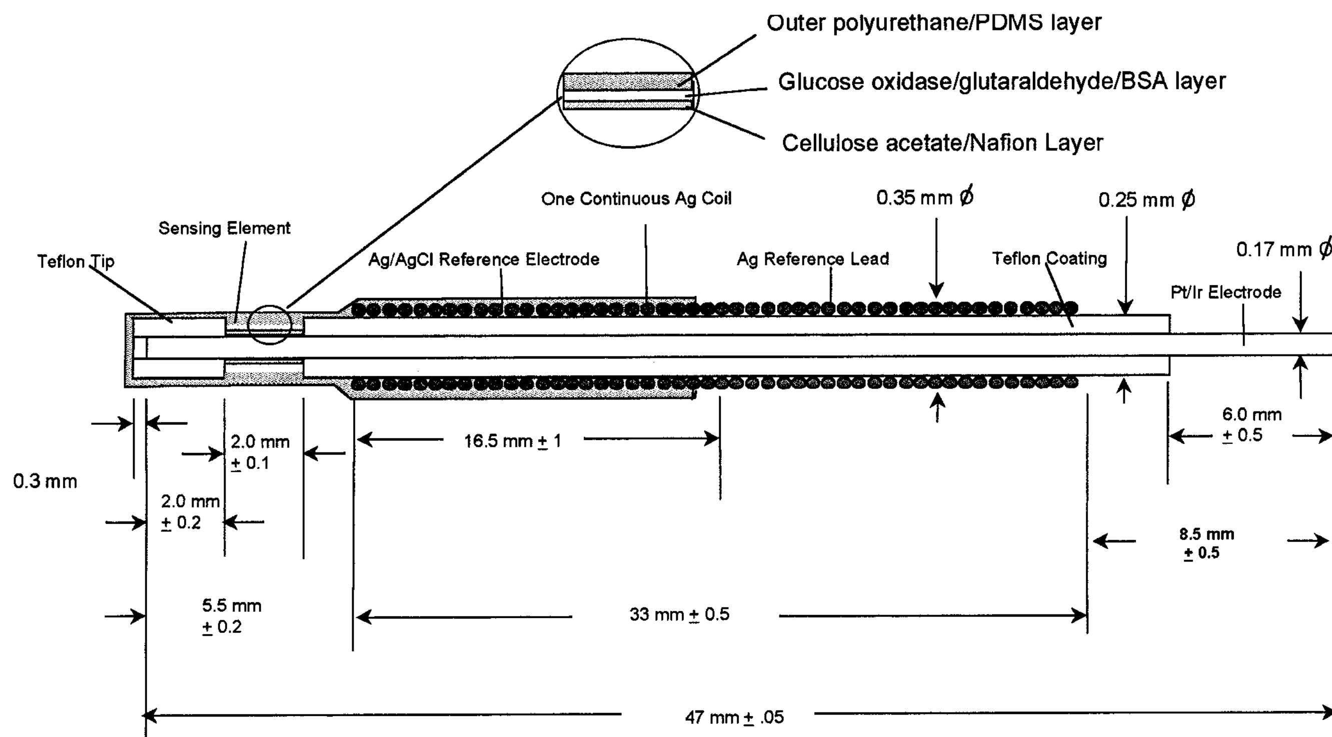

Figure 1. Schematic diagram of glucose biosensor used for glucose monitoring in humans (dimensions are in millimeters).

possibility that there may be synergistic effects on sensor response. Stability is difficult to evaluate because this necessarily requires periodic calibration of the implanted device. In the case of subcutaneous glucose monitoring, this is feasible but glucose is probably unique in this regard. Detection limits for most enzyme sensors are in the low micromolar range (0.1−1.0 μM) and are typically limited by the background current. Sensors have been fabricated in characteristic diameters down to 10 μm.[44] Such sensors may be required to have fast response times (seconds to subseconds for 90% response). This is challenging from a design point of view because the enzyme layer must be of finite thickness. However, if the layer is about 1 μm thick, then responses in the 10 ms range should be possible, based on the diffusion limit. So far, responses as short as 85 ms have been attained with a 0.27 μm diameter glucose sensor.[45]

IV. Continuous Monitoring of Blood Glucose

As noted above, significant effort has been expended by many groups toward the development of glucose sensors suitable for continuous monitoring. The reader is referred to a recent monograph on “Biosensors in the Body”.[46] We have focused attention on the development of a subcutaneously implantable glucose sensor, a key part of a continuous glucose monitoring system (CGMS). Such a system will, as a first step, be used to monitor blood glucose and provide diabetic patients with a continuous record of blood glucose fluctuations. In addition, the system will have the capability of detecting hypoglycemic (low blood sugar) events and so informing the patient. Eventually the sensor might be connected to an insulin pump; the resulting closed loop system might then be called an “artificial pancreas”. From the beginning it was clear that the implanted sensor had to be acceptable to the patient, and for this reason a size of 350 μm for the sensing element was chosen. The sensor is shown in Figure 1. It consists of a Pt−Ir wire coated with PTFE. Toward the distal end a portion of the insulation is removed to create a cavity, which forms the sensing element. On top of the electrode are three functional layers: the inner membrane, which ideally excludes all electroactive species except peroxide (S'_{red}, eq 2), an enzyme layer, and an outer membrane. The latter membrane has as its major function control of fluxes of glucose (S_{red}) and oxygen (S'_{ox}) into the enzyme layer. This membrane presents a substantial diffusional barrier to glucose transport and very little resistance to oxygen transport such that the response of the sensor is defined by the permeability of this membrane to glucose. This provides both the relative independence of response on oxygen and also renders the response to glucose linear up to at least 15−20 mM glucose. If a large excess of enzyme is present, then the response is independent of enzyme activity over a wide range, allowing for enzyme activity loss during operation. Finally, because the response is mass transfer rather than enzyme kinetically limited, the temperature coefficient is much smaller (2.5%/°C).[13a] The dependence of the response on oxygen is most serious at high glucose concentrations and where the outer membrane permeability to glucose is high.[20] In vitro and in vivo experiments in rats have established that at 15 mM glucose only a 5% change in sensor output is observed in passing from 150 to 7.5 mmHg of O_2. The tissue O_2 levels are estimated to be around 23 mmHg. Oxygen levels in tissue can be quite variable,

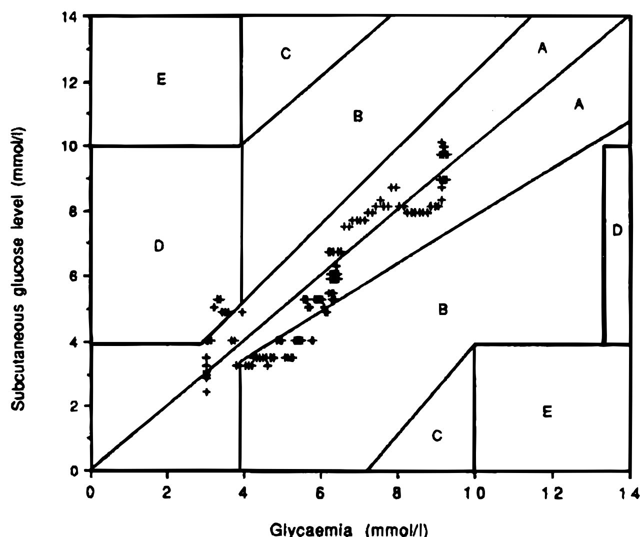

Figure 2. Clarke error grid analysis of subcutaneous glucose monitoring system performance in normal subjects ($n = 9$). (Reprinted with permission from ref 13c. Copyright 1993 Springer-Verlag.)

however, and care must be taken to ensure that sensor response is not oxygen dependent.[20]

As noted above, the inner membrane must be capable of excluding electroactive species such as ascorbate and urate. Acetaminophen, a widely employed analgesic, is often used as a "litmus test" for permselectivity. We have developed a composite inner membrane fabricated from alternate layers of cellulose acetate and Nafion, such that interferences can be effectively eliminated.[19a] This conclusion was supported by studies in rats and in humans[19a,b] indicating that three factors work in concert to minimize acetaminophen interference in practical applications: the permselectivity of the inner membrane, the relatively low levels in the tissue, and the rapid acetaminophen elimination after ingestion. A simple one-layer permselective coating has been developed to accomplish a high acetaminophen/peroxide exclusion ratio.[47]

Sensor performance is evaluated in several different ways, one such method being Clarke error grid analysis.[48] In this approach the "true" blood glucose value (as measured with a clinical analyzer) is plotted on the x-axis and the estimate obtained from the sensor on the y-axis. Perfect correlation would, of course, result in all points falling on a 45° line passing through the origin. Error grid analysis is an empirical approach that evaluates the *clinical* significance of deviations from ideality. This then addresses the question of whether a particular result would cause the patient to take incorrect action such as taking carbohydrate when they should be taking insulin. The zones of Figure 2 (one patient only) correspond to A = clinically accurate, B = clinically acceptable, and the remaining zones C, D, and E = progressively less acceptable and even dangerous. In this case, however, results obtained in nine different normal patients showed that 99% of the values fell in zones A and B.[13c] Special attention should be paid to the location of points corresponding to glucose concentrations less than about 5mM (normoglycemia). Of particular concern are values that fall in the D zone, corresponding to a sensor reading that is high when the actual blood glucose may be dangerously low.

A key issue in the use of in vivo sensors is their calibration. Because of the accuracy demanded of continuous monitoring systems, it is not feasible, at least so far, to use an in vitro precalibration as the basis for the sensor response function. Fortunately, it has been demonstrated that it is possible to use blood glucose, measured either with a clinical analyzer or a "fingerstick" system, to facilitate an in vivo calibration. Such calibrations are typically subject to the following assumptions: (1) The response of the sensor to glucose is linear over the relevant range of glucose concentrations (2−20 mM) and (2) the concentrations of glucose in the blood and interstitial fluid are either equal or constantly proportional during calibration and measurements. The former assumption is easily established by proper sensor design. The latter, on the other hand, is much more complicated and has been the subject of significant discussion.[7,12,49a,50−54] It is well established that the "apparent" ratio of blood/tissue glucose is not constant, and this observation may have at least three causes: (1) The intrinsic sensor dynamic response, (2) the kinetics of glucose transport between the vascular bed and the subcutaneous tissue, and (3) local perturbations of glucose and/or oxygen concentrations in the vicinity of the sensor. The latter two issues are probably the most problematic, and the feasibility of predicting tissue glucose concentrations has been recently examined.[43] It is not yet clear, however, that a kinetic model will be useful due to the complexities of glucose uptake by the subcutaneous tissue.[51] Relatively little attention has been paid to the latter issue, which is presented in a discussion of biocompatiblity below.

It is apparent, then, that a glucose sensor implanted in the subcutaneous tissue can be calibrated by measuring glucose concentration in the blood and assuming a tissue level that is proportional, typically equal. For short-term implants, patients will be required to measure their blood glucose level using a "fingerstick" system, devices whose performance (precision, accuracy) is certainly not optimal. Two general approaches are currently employed: the two-point and the one-point calibration. The two-point calibration is implemented by performing two blood glucose measurements, corresponding to two different sensor current outputs. The slope (sensitivity, S) and intercept (background current, I_0) are derived from the equation of the straight line. This approach requires the establishment of two plateaus, where the signal remains constant for a period of about 5 min.[49b] This approach is feasible but not very convenient for the diabetic patient. A second approach involves a one-point calibration, that is, in effect, a two-point calibration where the origin is assumed by definition to be one of the two points.[54] This method depends on the existence of a background current that is small (probably less than 10% of the signal) and with a relatively small standard deviation over the time that the calibration is considered valid. The effect of

multiple simultaneous sensor measurements on the reliability of estimation of glucose levels has also been examined.[54c]

The report of the results of a long-term prospective trial, the Diabetes Control and Complications Trial (DCCT), sharply focused attention on the importance of continuous glucose monitoring as a means for avoiding the increased incidence of hypoglycemia, a striking consequence of demonstrably beneficial intensive insulin therapy.[55] A consequence has been the qualified approval in the last year by the U.S. Food and Drug Administration of a continuous glucose monitoring system produced by Minimed.[56] It is designed to be implanted subcutaneously for approximately 3 days. After calibration, the sensor and accompanying pager-size unit generates a value for the tissue/blood glucose once per minute, which is then averaged over 5 min. These values are stored and subsequently downloaded by the physician at the end of the 3 days. A second system, developed by Cygnus, Inc., for which FDA approval is expected shortly, avoids implantation of the sensor by extracting glucose through the skin by reverse iontophoresis.[57] The glucose thus accumulated reacts with oxygen in a pad contacting the skin, producing peroxide that is then measured coulometrically with the iontophoretic current turned off. Because the glucose is accumulated over a period of time as is the charge during the subsequent measurement step, the overall process takes about 20 min. This system is designed to operate for about 12−15 h at a time, and the data will be accessed by a physician at the end of the test period. There are also several glucose monitoring systems based on microdialysis sampling of the subcutaneous tissue that have been developed in Europe, but their commercialization status is not known. In any case, an immediate objective of all of these systems will be to detect the onset of hypoglycemia and to provide a more comprehensive and effective approach to insulin therapy.

V. Microbiosensors for the Study of Neurological Processes

The pioneering work of Adams[58] and Wightman[59] in in vivo electrochemistry has demonstrated the utility of microsensors in the study of important issues in neurophysiology and neuropharmacology. Neurotransmitter release and uptake has been investigated in single cells, brain slices, and the intact brain. This work has demonstrated the advantages of microsensors in providing enhanced spatial and temporal resolution to the point of monitoring quantal release of neurotransmitters from individual cells.[59] This approach is feasible, however, because the neurotransmitters of interest (catecholamines and their metabolites, especially dopamine) are electroactive and, through the use of rapid scan cyclic voltammetry,[60−62] can be distinguished from endogenous electroactive species such as ascorbate. Unfortunately, many important neuromodulators are not intrinsically electroactive, and therefore, alternative methods must be found for monitoring them. Microdialysis[63−66] combined with discrete sampling, separation by HPLC or capillary electrophoresis, and subsequent determination can provide a comprehensive picture of metabolic processes occurring in the sampling region. Because the determination is not made in situ, a wide range of analytical methods may be applied to the measurement step that makes possible the simultaneous monitoring of several species. The disadvantage of microdialysis is its relatively poor temporal and spatial resolution, thus making difficult the characterization of processes in the second to subsecond time domain.

Table 1. Selectivity Ratios for Sensor Response to Glutamate vs Interferent

interferents	selectivity ratios[a]
L-ascorbic acid (AA)	>5000:1
uric acid (UA)	<1000:1
dopamine (DA)	23:1 (± 2.7, $n = 4$)
norepinephrine	47:1 (± 6.8, $n = 4$)
serotonin	28:1 (± 4.2, $n = 4$)
DOPAC	224:1 (± 29, $n = 4$)
HVA	1300:1 (± 221, $n = 4$)
MHPG	18:1 (± 2.2, $n = 4$)
5-HIAA	280:1 (± 50.4, $n = 4$)
L-tyrosine	58:1 (± 7.5, $n = 4$)
L-cysteine	146:1 (± 20, $n = 4$)
L-tryptophan	245:1 (± 41, $n = 4$)
L-aspartate	61:1 (± 7.3, $n = 4$)
L-glutamine	325:1 (± 42, $n = 4$)
glutathione	1600:1 (± 304, $n = 4$)
GABA	no response
catalase	no effect on the response to glutamate

[a] DOPAC, 3,4-dihydroxyphenyl acetic acid; HVA, 4-hydroxy-3-methoxyphenylacetic acid; MHPG, 3-methoxy-4-hydroxyphenylglycol; 5-HIAA, 5-hydroxyindolacetic acid. The selectivity ratio is calculated as the ratio of the glutamate current to the extrapolated current for the interferent at a concentration equal to that of glutamate (10 μM).

In recent years considerable progress has been made in the application of enzyme-based microbiosensors to neurophysiology measurements. Several reviews on this subject have recently appeared,[61b,67] and it is clear that relatively few applications have, so far, involved real-time measurements in the intact brain. It is ordinarily desired to make the sensor as small as possible to avoid tissue damage on implantation and to achieve fast response, ideally in the subsecond domain. To be sure that reliable results can be obtained from an enzyme microbiosensor, it is necessary to perform comprehensive in vitro tests. These include characterization of sensitivity, linearity, and stability, especially under continuous operation. Much less frequently examined are potential interferences, in particular those caused by endogenous electroactive species. Such a comprehensive study is illustrated by the results shown in Table 1 obtained for a glutamate sensor developed in our laboratory.[68] It is necessary to demonstrate not only that the presence of a particular interference does not change the signal to the analyte of interest, but also that there are no synergistic effects. This can be studied using the time-dependent response curves shown in Figure 3. In vitro evaluation is a necessary but not sufficient means of establishing sensor performance. The properties of the sensor in vivo are much more difficult to assess, and they can be different from those measured in vitro. Permselective membranes may be used to eliminate interferences,

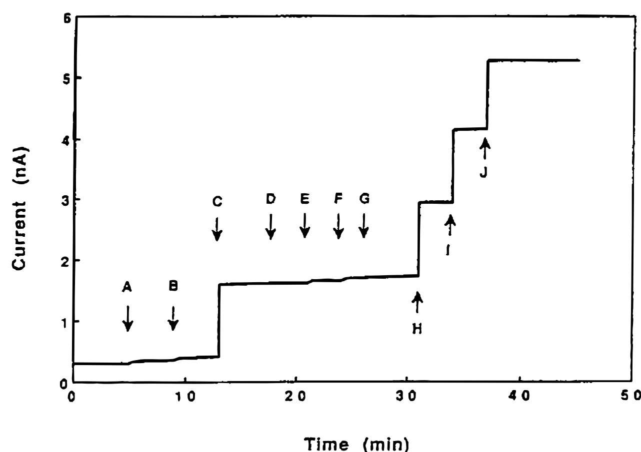

Figure 3. Effect of interferents on sensor response. Medium: 0.1 M phosphate-buffered saline, pH 7.4, 37 °C. The final concentrations at each addition were (A) AA, 200 μM; (B) DOPAC, 25 μM; (C) Glutamate (Glu), 10 μM; (D) UA, 50 μM; (E) DOPAC, 50 μM; (F) DA and 5-hydroxytryptamine (5-HT) together, 5 μM each; (G) AA, 500 μM; (H) Glu, 20 μM; (I) Glu, 30 μM; (J) Glu, 40 μM. (Reprinted with permission from ref 68. Copyright 1994 Elsevier Science B.V.)

but their required thickness can have a detrimental effect on sensor response time. Wightman and coworkers dealt with this problem in the context of direct electrochemistry through Nafion membranes,[69] but to our knowledge, such a treatment has not been applied to the more complex, multilayered enzyme-based devices. A major electroactive interference in the brain is ascorbate, which can be easily eliminated using ascorbate oxidase.[68,70] This enzyme reduces oxygen to water rather than peroxide, so that ascorbate elimination does not perturb peroxide levels. It may, however, perturb oxygen levels and thus affect the response to glutamate. It is accordingly essential to demonstrate that at the highest level of analyte envisioned, minimal changes in sensor output will be observed as oxygen partial pressure varies over normal physiological levels. Of particular concern are ischemic situations where the concentrations of glutamate or lactate are high but the levels of oxygen are low as noted above.

L-Glutamic acid is the most prevalent excitatory neurotransmitter in the mammalian brain, and its high concentration in extracellular fluid (ECF) caused by excessive release may play a major neurotoxic role in a wide range of neurological disorders. Thus, sensors capable of monitoring the release and uptake of glutamate in real time can contribute to the better understanding of glutamate- induced physiological and pathological states in the central nervous system (CNS). There have been a number of recent reports of in vivo measurements of glutamate, all employing amperometric detection and immobilized glutamate oxidase.[71−73] Our sensor has a response time of about 1 s, measured in vitro in a flow injection experiment with a detection limit for glutamate of less than 2 μM.[68] It employs an inner layer of Nafion/cellulose acetate and ascorbate oxidase to eliminate interferences. When the sensor is placed in the dentate gyrus of the hippocampus of an anesthetized rat, it is possible to measure glutamate release and uptake resulting from local KCl stimulation or remote electrical stimulation. In this latter situation a bipolar stimulating electrode is placed in the angular bundle of the perforant pathway (known as the glutamatergic axonal pathway). Biphasic fluctuations in ECF glutamate concentration are observed as the neurotransmitter is released and subsequently taken up by both neurons and glial cells.

Microdialysis and the use of a microbiosensor have been combined to produce a monitoring system. In one instance the microbiosensor monitors the microdialysis perfusate,[74] while in the other, the sensor is located within the dialysis probe itself.[75] The continuous measurement of glutamate efflux in the striatum of a freely moving rat following a tail pinch stimulation and other recent applications have been reported.[76,77]

Choline, another important substrate in both the peripheral and central nervous system, has also been monitored in the brain using an enzyme-modified amperometric microelectrode by immobilization of horseradish peroxidase (HRP) and choline oxidase within a redox hydrogel. The latter mediates electron transfer between HRP and the electrode. The sensor is built on a 10 μm carbon fiber and has a response time of about 15 s.[44] An acetylcholine sensor was created by adding acetylcholinesterase to the redox hydrogel film.[44] Wightman and co-workers[78] used TTF^+TCNQ^- as the mediator in the preparation of choline and acetylcholine microbiosensors.

Under normal physiological conditions glucose is the major source of energy powering the mammalian brain. Glucose is transported into brain across the blood brain barrier (BBB) by a saturable process of facilitated mass transfer. Previous studies using the 2-deoxyglucose method confirm that there is a tight coupling between phosphorylation and glucose transport into the brain which is correlated with glucose utilization and regional cerebral blood flow (rCBF).[79] Because of the key role played by rCBF, studies must be carried out in the intact brain and fast sensor response is required because classical steady-state assumptions are not appropriate.

The in vivo measurement of glucose variations was first demonstrated by Silver[80] using a Pt-coated glass micropipet (0.1 μm diameter) on which glucose oxidase was adsorbed. More recently, several sensors have been developed for brain glucose measurements.[41,81−85] We have developed a sensor for brain glucose measurements[86] which is similar in construction but smaller than that shown in Figure 1 (diameter 110 μm) with a response time of 5 s for 90% maximum response. The sensor shows excellent stability and selectivity, especially for ascorbate. Ascorbate elimination is far simpler than for the glutamate sensor because the concentrations of the analyte are 3 orders of magnitude higher. On the other hand, more oxygen is required to “run” the sensor and care must be taken to be sure that fluctuations in the cosubstrate are not affecting sensor response. Simultaneous measurement of plasma (blood) and interstitial brain glucose showed the same trends as the blood glucose was altered by ip glucose and insulin injection. However, brain glucose levels are always

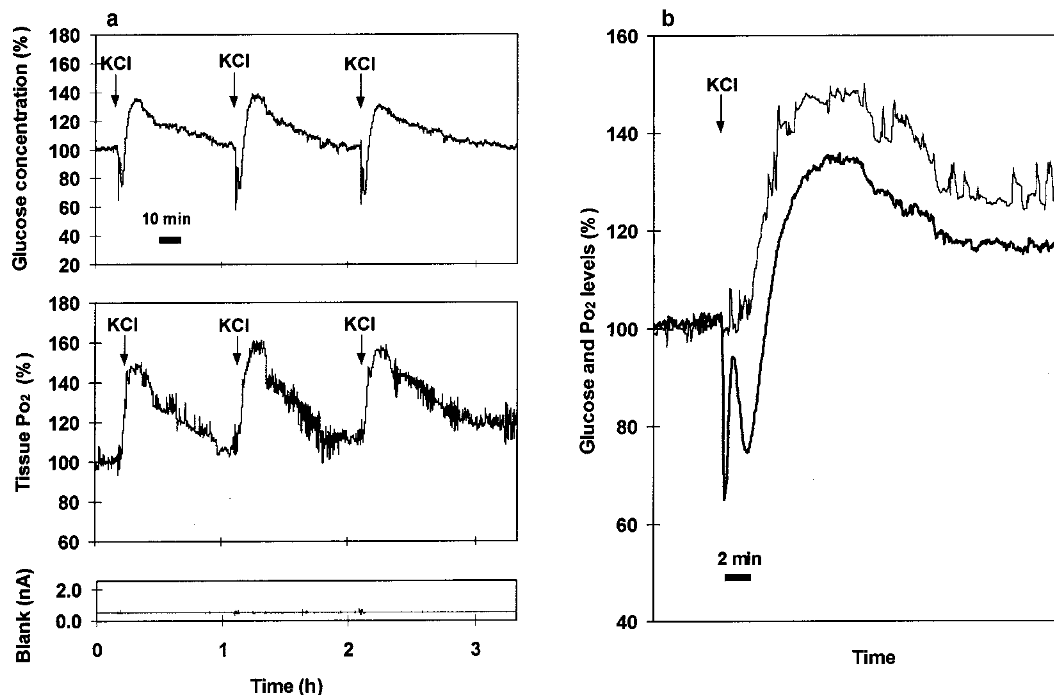

Figure 4. (a) Typical original profiles show simultaneous response of a glucose sensor (upper), an oxygen electrode (middle), and a blank sensor without glucose oxidase (lower), stereotaxically positioned in the dentate gyrus of rat brain, to local microinjection of KCl (5 nmol in 0.05 μL of saline). Three stimulations, at about 1 h intervals, were made in an anesthetized rat from 15 similar experiments, showing good reproducibility. After each stimulation, the extracellular glucose returned to the original level. (b) Expansion of one stimulation from panel a showing detailed multiphasic alterations in extracellular glucose (thick line) and oxygen (thin line) levels during perturbation caused by KCl. (Reprinted with permission from ref 86. Copyright 1997 International Society for Neurochemistry.)

much lower than those in the blood. Basal glucose levels are currently the subject of debate as we obtain 2.6 mM in normoglycemic anesthetized rats. This value is in agreement with previous findings of 2.4 mM obtained by Silver and Erecinska[82] and with results obtained after focused microwave irradiation[87,88] and NMR.[89] These results are all clearly higher than the 0.47 mM obtained by Fellows et al.[90] and 0.35 mM by Lowry[85] in awake animals. However, administration of anesthesia can significantly perturb levels of analytes being monitored.[61a]

VI. Multidimensional Biosensor Measurements

As technology becomes available to facilitate development of sensor arrays, it becomes increasingly possible to simultaneously measure the time-dependent variation in several analytes. This is very important in complex biological systems because the correlated time dependence often provides clues as to how regulation is accomplished. Simultaneous measurements in our laboratory of glucose and oxygen in the dentate gyrus of the rat hippocampus demonstrated, surprisingly, that transient regional changes in extracellular glucose occur without obvious decrease in oxygen levels following neuronal stimulation. The utility of this approach is illustrated in Figure 4. An initial transient, rapid (10−13 s) glucose decrease of up to 34% is observed in response to an acute energy demand (KCl stimulation). The nearly simultaneous increase in oxygen levels is suggestive of an increase in rCBF, resulting as well in increased delivery of glucose. Similar trends are observed when electrical neuronal stimulation is accomplished through the perforant path.

The findings of this study suggest that transient limitations of glucose transport across the BBB may occur upon acute energy demands, implying that traditional understanding of dynamic regulation of cerebral metabolism may need to be reconsidered. This finding is also consistent with observations in other groups using biosensors and microdialysis.[82,85,90] Given this situation, the key question becomes "How is regional energy homeostasis maintained for neuronal functional activity?" Part of the answer to this question comes from expanding simultaneous measurements to include lactate.

Lactate has long been considered a harmful metabolic byproduct that degrades the performance of athletes and may contribute to the damage of the CNS in pathological situations involving acidosis such as hypoxia, ischemia, seizures, and trauma. However, compelling in vitro evidence from recent studies using brain slices and cell cultures has strongly implicated lactate as an alternative CNS energy source. There have, however, been few studies involving lactate monitoring with brain biosensors.[91,92]

We have constructed a lactate sensor using the same strategy as that for brain glucose but using lactate oxidase.[91] Three electrodes (glucose, lactate, and oxygen) were simultaneously implanted in the dentate gyrus within 50−100 μm of each other. Extracellular levels of the three species were simul-

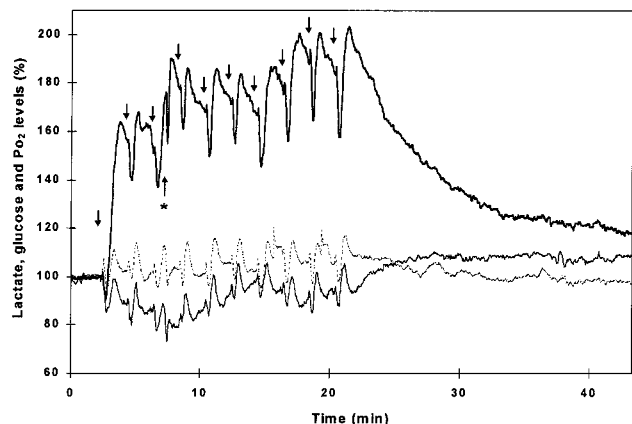

Figure 5. Typical original profiles show the time course and dynamic relationships of local extracellular lactate (thick solid line), glucose (thin solid line), and Po_2 (dotted line) levels during a sequence of 5-s electrical stimulations of the perforant path with 2-min rest intervals. Simultaneous real-time measurements were carried out in the dentate gyrus of the rat brain using miniaturized sensors with rapid response time. The arrows indicate the time of each repeated stimulation. (The arrow with asterisk marks a spontaneous transient drop of lactate level. The reason for the drop was unclear.) (Reprinted with permission from ref 91. Copyright 1997 International Society for Neurochemistry.)

taneously measured following a sequence of 5-s stimulations with 2 min intervals. Figure 5 shows the rich body of information that can be extracted from such an experiment. Repeated stimulations resulted in sustained high levels of extracellular lactate that returned to baseline only when the stimulation was stopped. Mean cellular levels of lactate and glucose always varied in opposite directions following exactly the same time course. There was always a deeper rapid transient depletion up to 28% in lactate following each subsequent stimulation from the sustained mean level of lactate which was between 180% and 200% above the basal level, suggesting that lactate is being preferentially consumed to meet energy demands. As lactate depletion on stimulation increases, depletion of glucose becomes much less pronounced. These results are supported by ample in vitro observations demonstrating that astrocytes take up glucose and export large quantities of lactate to the medium for neuronal use and that glutamate stimulates astrocytic glycolysis to produce lactate.[93]

The results of this study give a real-time dynamic picture of changes in *extracellular* concentrations of key components in regional energy homeostasis in the brain. The fact that the measured oxygen levels are above the basal level supports the idea that lactate is produced by glycolysis under overall *aerobic* conditions and that under acute energy requirements lactate is the preferred energy source. Acute energy demand creates a local fuel "reservoir" behind the BBB resulting from elevated levels of lactate. This model is supported by in vitro observations that have demonstrated glutamate uptake into astrocytes in cultured cell preparations stimulates glucose utilization and lactate production via an increase in aerobic glycolysis.[94,95]

The value of simultaneous measurements has triggered the development of sensor arrays where the geometrical relationships between the various sensing elements are well defined. For example, Xin and Wightman[96] carried out simultaneous detection of Ca^{2+} and catecholamines by deposition of a Ca^{2+}-sensitive fluorescent dye on the surface of a 5 μm carbon fiber electrode. It was then possible to detect micromolar concentrations of catecholamines and 100 nM concentrations of Ca^{2+} using this system. Differences in electrochemical properties have made it possible to simultaneously detect histamine and 5-hydroxytryptamine using fast scan cyclic voltammetry.[97] Optical sensors, though not discussed in this review, are destined to play a larger role in in vivo measurements. Submicrometer fiber optic sensors for measuring glucose have been developed.[98] Even more interesting is the possibility of imaging a biological surface using a coherent array architecture created by a bundle of optical fibers. This has been demonstrated using an acetylcholine biosensor, which measures fluorimetrically the change in pH resulting from acetylcholine hydrolysis.[99]

VII. Biocompatibility

When a biosensor is implanted in an in vivo system, the host medium is perturbed. Biocompatibility thus becomes a question of the extent to which the resulting interactions affect the proper function of the sensor and also the extent to which the implant modifies the function of the host. There are two separate but related processes that must be considered: tissue/sensor interactions resulting from a cascade of reactions called the inflammatory response (wound healing) and blood interactions eventually resulting in thrombus formation. For many applications implantation of sensors in the vascular bed is avoided because of the danger of thromboembolism. Thus, the interstitial fluid of the subcutaneous tissue has so far proven to be the most suitable site for continuous measurement of analytes such as glucose. When a sensor is implanted in this environment, tissue is disrupted and capillaries damaged. Although the process of hemostasis and the subsequent tissue response are understood in general terms,[100] considerably less is known about how these processes affect sensor performance.

Qualitatively, there appear to be three overlapping time domains important to biosensor function: rapid sensitivity loss (minutes-hours), acute inflammatory response (days), and foreign body capsule formation (weeks). The most rapid of these processes occurs in a matter of several hours during which time sensors can lose 50% or more of their in vitro sensitivity. We have found the process to be reversible.[13d] Removal of the sensor from the tissue and rapid calibration in buffer solution yields the same sensitivity as that determined in vivo, but if the sensor is allowed to remain in the buffer for several hours, the original in vitro sensitivity is attained. The exact cause of the sensitivity loss is not known, but it should not be concluded that proteins are the prime source of the problem. Because the extent of the loss is unpredictable, it is necessary to calibrate sensors in vivo. There

have been several strategies proposed for minimizing such effects including suppression of protein adsorption on the sensor surface.[101] Hydrogels (poly(ethylene glycol)s, polymethacrylates, poly(vinyl alcohol), etc.) tend to show reduced tissue interaction.[102] Rational design of outer membranes is confounded by the complexity of the observed interactions. Surface properties of implant materials have been linked to cell–implant interactions through surface group properties,[103] surface free energy,[104,105] surface charge, and surface charge density.[101,102] Biocompatibility is sometimes linked to cell response by such assays as human monocyte activation and human fibroblast proliferation.[108] Histology of implants in rats is also studied. It must be emphasized that outer membranes designed to render the sensor more "biocompatible" must not interfere adversely with the transport to the sensing layer of the analyte and cofactors such as oxygen.

The second stage of interaction, that of the acute inflammatory response, has been extensively studied by Anderson and co-workers[109] A series of reactions leading from blood monocytes to macrophages and eventually to foreign body giant cells is of concern especially for sensors implanted for periods of 1–2 weeks. Using the so-called "cage implant method", Anderson's group characterized various materials with respect to their ability to evoke inflammatory response by analyzing the exudate localized within a wire cage implanted in the dorsal subcutis of a rat.[110] An aspect of the acute inflammatory response that has received relatively little attention is the extent to which the interaction of macrophages with the surface of the implanted sensor affects sensor response. It is known that macrophages consume oxygen and glucose and produce superoxide and peroxide, a situation that could lead to substantial perturbation of the concentrations of relevant species at the sensor surface. Microdialysis glucose recovery studies in subcutaneous adipose tissue of humans over a period of 3 weeks suggest that the decreasing inflammatory response over the first 10 days may indeed lower the glucose concentration at the probe surface.[111] Moreover, because of the oxidative processes occurring, the pH could be as low as 3.5–3.7.[112]

The third stage of the wound healing process results in the formation of a fibrotic capsule around the implant. This could potentially interfere seriously with the delivery of analyte to the sensor surface. Several groups are attempting to deal with this problem by developing textured surfaces that encourage vascularization of the sensor surface and the surrounding tissue, thus ensuring that the capillaries can continue to deliver the glucose and oxygen needed for proper sensor operation.[113] This question is of particular concern in the case of long-term implants (several months or more).

The bulk properties of implant materials also have to be considered. For example, leachates from the sensor manufacture such as organic solvents or reagents used in polymerization as well as mediators[113] must be considered to be potentially toxic. For long-term implants, the chemical stability of polymeric materials must also be considered.[110] Further, standard toxicology tests must be performed to establish that the sensor is safe for implantation if used in humans (Ames test (mutagenicity), dermal allergenicity, cytotoxicity, intracutaneous irritation, and test of necrosis). Sensors must also be prepared under sterile conditions.

Despite the complexity of living systems and the problems associated with in vivo measurements using biosensors, significant progress has been made in improving reliability and extending capabilities to higher sensitivity and selectivity, and faster response time.

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IX. References

(1) Clark, L. C., Jr. *Trans. Am. Soc. Artif. Intern. Organs* **1956**, *2*, 41–48.
(2) Clark, L. C., Jr.; Lyons, C. *Ann. NY Acad. Sci.* **1962**, *102*, 29–45.
(3) Clark, L. C., Jr. In *Biosensors: Fundamentals and Applications*; Turner, A. P. F., Karube, I., Wilson, G. S., Eds.; Oxford University Press: New York, 1987.
(4) Shichiri, M.; Kawamori, R.; Goriya, Y.; Yamasaki; Nomura, M.; Hakui, N.; Abe, H. *Diabetologia* **1983**, *24*, 179–184. Hashiguchi, Y.; Sakakida, M.; Nishida, K.; Uemura, T.; Kajiwara, K. I.; Shichiri, M. *Diabetes Care* **1994**, *17*, 387–396.
(5) Claremont, D. J.; Sambrook, I. E.; Penton, C.; Pickup, J. C. *Diabetologia* **1986**, *29*, 817–821.
(6) Churchous, S,; Mullen, W.; Battersby, C. M.; Vadgama, P. *Biosensors* **1986**, *2*, 325–333.
(7) Fischer, U.; Ertle, R.; Abel, P.; Rebrin, K.; Brunstein, E.; Hahn von Dorsche, H.; Freyse, E. J. *Diabetologia* **1987**, *30*, 940–945.
(8) Armour, J. C.; Lucisano, J. Y.; McKean, B. D.; Gough, D. A. *Diabetes* **1990**, *39*, 1519–1526.
(9) Koudelka, M.; Rohner-Jeanrenaud, F.; Terrettaz, J.; Bobbioni-Harsch, E.; de Rooij, N. F.; Jeanrenaud, B. *Biosens. Bioelectron.* **1991**, *6*, 31–36.
(10) Johnson, K. W.; Mastrototaro, J. J.; Howey, D. C.; et al. *Biosens. Bioelectron.* **1992**, *7*, 709–714. Johnson, K. W.; Allen, D. J.; Mastrototaro, J. J.; Morff, R. J.; Nevin, R. S. In *Diagnostic Biosensor Polymers*; Usmani, A. M., Akmal, N., Eds.; ACS Symposium Series 556; American Chemical Society:, Washington, DC, 1994.
(11) Moussy, F.; Harrison, D. J.; O'Brien, D. W.; Rajotte, R. V. *Anal. Chem.* **1993**, *65*, 2072–2077.
(12) Pfeiffer, E. F.; Meyerhoff, C.; Bischof, F.; Keck, F. S.; Kerner, W. *Horm. Metab. Res.* **1993**, *25*, 121–124; Sternberg, F.; Meyerhoff, C.; Mennel, F. J.; Hoss, U.; Mayer, H.; Bischof, F.; Pfeiffer, E. F. *Horm. Metab. Res.* **1994**, *26*, 523–525.
(13) (a) Bindra, D. S., Zhang, Y.; Wilson, G. S.; Sternberg, R.; Thévenot, D. R.; Moatti, D.; Reach, G. *Anal. Chem.* **1991**, *63*, 6–669. (b) Moatti-Sirat, D.; Capron, F.; Poitout, V.; Reach, G.; Bindra, D. S.; Wilson, G. S.; Thévenot, D. R. *Diabetologia* **1992**, *35*, 224–230. (c) Poitout, V.; Moatti-Sirat; Reach, G.; Zhang, Y.; Wilson, G. S.; Lemonnier, F.; Klein, J.-C. *Diabetologia* **1993**, *36*, 658–663. (d) Thomé-Duret, V.; Gangnerau, M. N.; Zhang, Y.; Wilson, G. S.; Reach, G. *Diabetes Metab.* **1996**, *22*, 174–178. (e) Thomé-Duret, V.; Aussedat, B.; Reach , G.; Gangnerau, M. N.; Lemonnier, F.; Klein, J.-C.; Zhang, Y.; Hu, Y.; Wilson, G. S. *Metab., Clin. Exp.* **1998**, *47*, 799–803.
(14) Gilligan, B. J.; Shults, M. C.; Rhodes, R. K.; Updike, S. J. *Diabetes Care* **1994**, *17*, 882–887. Updike, S. J.; Shults, M. C.; Rhodes, R. K.; Gilligan, B. J.; Luebow, J. O.; von Heimburg, D. *ASAIO J.* **1994**, 157–163.
(15) Ward, W. K.; Wilgus, E. S.; Troupe, J. E. *Biosens. Bioelectron.* **1994**, *9*, 423–428.
(16) Csöregi, E,; Quinn, C. P.; Schmidtke, D. W.; Lindquist, S. E.; Pishko, M. V.; Ye, L.; Katakis, I.; Hubbell, J. A.; Heller, A. *Anal. Chem.* **1994**, *66*, 3131–3138.
(17) Atanasov, P.; Yang, S.; Salehi, C.; Ghindilis, A. L.; Wilkins, E. *Med. Eng. Phys.* **1996**, *18*, 632–640.
(18) Tamada, J. A.; Bohannon, N. J. V.; Potts, R. O. *Nat. Med.* **1995**, *1*, 1198–1201. Rao, G.; Guy, R. H.; Glikfeld, P.; LaCourse, W.

R.; Leung, L.; Tamada, J. A.; Potts, R. O.; Azimi, N. *Pharm. Res.* **1995**, *12*, 1869–1873.
(19) (a) Zhang, Y.; Hu, Y.; Wilson, G. S.; Moatti-Sirat, D.; Poitout, V.; Reach, G. *Anal. Chem.* **1994**, *66*, 1183–1188. (b) Moatti-Sirat, D.; Poitout, V.; Thomé, V.; Gangnerau, M. N.; Reach, G.; Zhang. Y.; Hu, Y.; Wilson, G. S.; Lemonnier, F.; Klein, J.-C. *Diabetologia* **1994**, *37*, 610–616.
(20) Zhang. Y.; Wilson, G. S. *Anal. Chim. Acta* **1993**, *281*, 513–520.
(21) Lucisano, J. Y.; Gough, D. A. *Anal. Chem.* **1988**, *60*, 1272–1281. Armour, J. C.; Lucisano, J. Y.; McKean, B. D.; Gough, D. A. *Diabetes* **1990**, *39*, 1519–1526. Gough, D. A.; Armour, J. C.; Baker, D. A. *Diabetologia* **1997**, *40 (Suppl. 2)*, S102–107.
(22) Pan, S.; Arnold, M. A. *Anal. Chim. Acta* **1993**, *283*, 663–671.
(23) Csöregi, E.; Gorton, L.; Marko-Varga, G. *Anal. Chim. Acta* **1993**, *273*, 59–70.
(24) Zhao, J.; Henkens, R. W.; Stonehuerner, J.; O'Daly, J. P.; Crumbliss, A. L. *Electroanal. Chem.* **1992**, *327*, 109–119.
(25) Degani, Y.; Heller, A. *J. Phys. Chem.* **1987**, *91*, 1285–1289. Degani, Y.; Heller, A. *J. Phys. Chem.* **1988**; *J. Am. Chem. Soc.* **1988**, *110*, 2615. Heller, A. *J. Am. Chem. Soc.* **1990**, *23,* 128–134.
(26) Badia, A.; Carlini, R.; Fernandez, A.; Battaglini, F.; Mikkelsen, S. R.; English, A. M. *J. Am. Chem. Soc.* **1993**, *115*, 7053–7060.
(27) Battaglini, F.; Koutroumanis, M.; English, A. M.; Mikkelsen, S. R. *Bioconjugate Chem.* **1994**, *5*, 430–435.
(28) Kenausis, G.; Taylor, C.; Katakis, I.; Heller, A. *J. Chem. Soc., Faraday Trans.* **1996**, *92*, 4131–4136.
(29) Ohara, T. J.; Rajagopalan, R.; Heller, A. *Anal. Chem.* **1994**, *66*, 2451–2457.
(30) Willner, I.; Heleg-Shabtai, V.; Blonder, R.; Bòchmann, A. F.; Heller, A. *J. Am. Chem. Soc.* **1996**, *118*, 10321–10322.
(31) Cass, A. E. G.; Davis, G.; Francis, G. D.; Hill, H. A. O.; Aston, W. J.; Higgins, I. J.; Plotkin, E. V.; Scott, L. D. L.; Turner, A. P. F. *Anal. Chem.* **1984**, *56*, 667. Henning, T. P.; Cunningham, D. D. In *Commercial Biosensors: Applications to Clinical, Bioprocess, and Environmental Samples*; Ramsay, G., Ed.; Wiley and Sons: New York, 1998; Chapter 1.
(32) Bu, H.; English, A. M.; Mikkelsen, S. R. A*nal. Chem.* **1996**, *68*, 3951–3957. Calvo, E. J.; Danilowicz, C.; Diaz, L. *J. Chem. Soc., Faraday Trans.* **1993**, *89*, 377–384.
(33) Atanasov, P.; Kaisheva, A.; Gamburzev, S.; Iliev, I.; Bobrin, S. *Electroanalysis* **1993**, *5*, 91–97.
(34) Kulys, J.; Buch-Rasmussen, T.; Bechgaard, K.; Razumas, V.; Kazlauskaite, J.; Marcinkeviciene, J.; Christensen, J. B.; Hansen, H. E. *J. Mol. Catal.* **1994**, *91*, 407–420.
(35) Persson, B.; Lee, H. S.; Gorton, L.; Skotheim, T.; Bartlett, P. *Electroanalysis* **1995**, *7*, 935–940.
(36) Cenas, N. K.; Kulys, J. *Bioelectrochem. Bioenergetics* **1981**, *8*, 103–113.
(37) Hill, B. S.; Scolari, C. A.; Wilson, G. S. *J. Electroanal. Chem.* **1988**, *252*, 125–138.; Albery, W. J.; Bartlett, P. N.; Bycroft, M.; Craston, D. W. *J. Electroanal. Chem.* **1987**, *218*, 119–126.
(38) Bartlett, P. N.; Booth, S.; Caruana, D. J.; Kilburn, J. D.; Santamaria, C. *Anal. Chem.* **1997**, *69*, 734–742.
(39) Khan, G. F.; Ohwa, M.; Wernet, W. *Anal. Chem.* **1996**, *68*, 2939–2945.
(40) Wang, J.; Lu, F. *J. Am. Chem. Soc.* **1998**, *120*, 1048.
(41) Boutelle, M. G.; Stanford, C.; Fillenz, M.; Albery, W. J.; Bartlett, P. N. *Neurosci Lett.* **1986**, *72*, 283–288.
(42) Schmidtke, D. W.; Heller, A. *Anal. Chem.* **1998**, 78, 2149–2155.
(43) Schmidtke, D. W.; Freeland, A. C.; Heller, A.; Bonnecaze, R. T. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 294–299. Quinn, C. P.; Pishko, M. V.; Schmidtke, D. W.; Ishikawa, M.; Wagner, J. G.; Raskin, P.; Hubbell, J. A.; Heller, A. *Am. J. Physiol.* **1995**, *269*, E155–E161.
(44) Garguilo, M. G.; Michael, A. C. *J. Neurosci. Methods* **1996**, *70*, 73–82. Garguilo, M. G.; Michael, A. C. *Anal. Chim. Acta* **1995**, *307*, 291–299.
(45) Meyerhoff, J. B.; Ewing, M. A.; Ewing, A. G. *Electroanalysis* **1999**, *11*, 308–312. Fraser, D. M. *Biosensors in the Body: Continuous In-vivo Monitoring*; J. Wiley and Sons: New York, 1997.
(46) Fraser, D. M. *Biosensors in the Body: Continuous In-vivo Monitoring*; J. Wiley and Sons: New York, 1997.
(47) Benmakroha, Y.; Christie, I.; Desai, M.; Vadgama, P. *Analyst* **1996**, *121*, 521–526.
(48) Clarke, W. L.; Cox, D.; Gonder-Frederick, L. A.; Carter, W.; Pohl, S. L. *Diabetes Care* **1987**, *10*, 622–628.
(49) (a) Thomé-Duret, V.; Reach, G.; Gangnerau, M. N.; Lemonnier, F.; Klein, J. C.; Zhang, Y.; Hu, Y.; Wilson, G. S. *Anal. Chem.* **1996**, *68*, 3822–3826. (b) Aussedat, B.; Thomé-Duret, V.; Reach, G.; Lemonnier, F.; Klein, J.-C.; Hu, Y.; Wilson, G. S. *Biosens. Bioelectron.* **1997**, *12*, 1061–1071.
(50) Sternberg, F.; Meyerhoff, C.; Mennel, F. J.; Bishof, F.; Pfeiffer, E. F. *Diabetologia* **1996**, *39*, 609–612. Moberg, E.; Hagström-Toft, Arner, P.; Bolinder, J. *Diabetologia* **1997**, *40*, 1320–1326.
(51) Aussedat, B.; Dupire-Angel, M.; Gifford, R.; Klein, J.-C.; Wilson, G. S.; Reach, G. *Am. J. Physiol.* **2000**, *278*, E716–E728.
(52) Bolinder, J.; Sjoberg, S.; Arner, P. *Diabetologia* **1996**, *39*, 845–853. Moberg, E.; Hagström-Toft, E.; Arner, P.; Bolinder, J. *Diabetologia* **1997**, *40*, 1320–1326.
(53) Baker, D. A.; Gough, D. A. *Anal. Chem.* **1996**, *68*, 1292–1297.
(54) (a) Csöregi, E.; Schmidtke, D. W.; Heller, A. *Anal. Chem.* **1995**, *67*, 1240–1244. (b) Schmidtke, D. W.; Pishko, M. V.; Quinn, C. P.; Heller, A. *Anal. Chem.* **1996**, *68*, 2845–2949. (c) Schmidtke, D. W.; Heller, A. *Anal. Chem.* **1998**, *70*, 2149–2155.
(55) The Diabetes Control and Complications Trial Research Group. *N. Engl. J. Med.* **1993**, *329*, 977–986. The Diabetes Control and Complications Trial Research Group. *Diabetes* **1997**, *46*, 271–286. Home, P. G. In *International Textbook of Diabetes Mellitus*; Alberti, K. G. M. M., Zimmet, P., DeFronzo, R. A., Keen, H., Eds.; John Wiley: New York, 1997; Chapter 40.
(56) Mastrototaro, J. J.; Levy, R.; Georges, L.-P.; White, N.; Mestman, J. *Diabetes* **1998**, *47* (*Suppl. 1)*, 238 (abstract). Rebrin, K.; Van Antwerp, W. P.; Mastrototaro, J. J. *Diabetes* **1997**, *46 (Suppl. 1)*, 611 (abstract).
(57) Kurnik, R. T.; Berner, B.; Tamada, J.; Potts, R. O. *J. Electrochem. Soc.* **1998**, *145*, 4119–4125. Kurnik, R. T.; Oliver, J. J.; Waterhouse, S. R.; Dunn, T.; Jayalakshmi, Y.; Lesho, M.; Lopatin, M.; Tamada, J.; Wei, C.; Potts, R. O. *Sens. Actuators B* **1999**, *B60*, 19–26. Tamada, J. A.; Garg, S.; Jovanovic, L.; Pitzer, K. R.; Fermi, S.; Potts, R. O. *JAMA* **1999**, *282*, 1839–1844. Garg, S.; Potts, R. O.; Ackerman, N. R.; Fermi, S. J.; Tamada, J. A.; Chase, F. P. *Diabetes Care* **1999**, *22*, 1708–1714.
(58) Kissinger, P. T.; Hart, J. B.; Adams, R. N. *Brain Res.* **1973**, *55*, 209–213. Adams, R. N. *Prog. Neurobiol.* **1990**, *35*, 297–311.
(59) Wightman, R. M.; Jankowski, J. A.; Kennedy, R. T.; Kawagoe, K. T.; Schroeder, T. J.; Leszczyszyn, D. J.; Near, J. A.; Diliberto, E. J., Jr.; Viveros, O. H. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 10754–10758.
(60) Kawagoe, K. T.; Zimmerman, J. B.; Wightman, R. M. *J. Neurosci. Methods* **1993**, *48*, 225–240.
(61) (a) O'Neill, R. D.; Lowry, J. P.; Mas, M. *Crit. Rev. Neurobiol.* **1998**, *12*, 69–127. (b) O'Neill, R. D. *Analyst* **1994**, *119*, 767–779.
(62) Boulton, A. A.; Baker, G. B.; Adams, R. N. *Voltammetric Methods in Brain Systems*; Humana Press: New Jersey, 1995.
(63) Benveniste, H.; Drejer, J.; Schousboe, A.; Diemer, N. H. *J. Neurochem.* **1984**, *43*, 1369–1374. Benveniste, H.; Diemer, N. H. *Acta Pathol. (Berl)* **1987**, *74*, 234–238.
(64) Justice, J. B., Jr. *J. Neurosci. Methods* **1993**, *48*, 263–276.
(65) Kehr, J. *J. Neurosci. Methods* **1993**, *48*, 251–261.
(66) Humpel, C.; Ebendal, T.; Olson, L. *J. Mol. Med.* **1996**, *74*, 523–524.
(67) Pantano, P.; Kuhr, W. G. *Electroanalysis* **1995**, *7*, 405–416.
(68) Hu, Y.; Mitchell, K. M.; Albahadily, F. N.; Michaelis, E. K.; Wilson, G. S. *Brain Res.* **1994**, *659*, 117–125.
(69) Bunin, M. A.; Wightman, R. M. *Methods Enzymol.* **1998**, *296*, 689–707.
(70) Nagy, G.; Rice, M. E.; Adams, R. N. *Life Sci.* **1982**, *31*, 2611–2616.
(71) Tamiya, E.; Karube, I. *Ann. N.Y. Acad. Sci.* **1992**, *672*, 272–277.
(72) Zilkha, E.; Koshy, A.; Obrenovitch, T. P. *Anal. Lett.* **1994**, *27*, 453–473.
(73) Ryan, M. R.; Lowry, J. P.; O'Neill, R. D. *Analyst* **1997**, *122*, 1419–1424.
(74) Zilkha, E.; Obrenovitch, T. P.; Koshy, A.; Kusakabe, H.; Bennetto, H. P. *J. Neurosci. Methods* **1995**, *60*, 1–9.
(75) Albery, W. J.; Boutelle, M. G.; Galley, P. T. *J. Chem. Soc., Chem. Commun.* **1992**, *12*, 900–901.
(76) Walker, M. C.; Galley, P. T.; Errington, M. L.; Shorvon, S. D.; Jefferys, J. G. R. *J. Neurochem.* **1995**, *65*, 725–731.
(77) Asai, S.; Iribe, Y.; Kohno, T.; Ishikawa, K. *Neuroreport* **1996**, *7*, 1092–1096.
(78) Xin, Q.; Wightman, R. M. *Anal. Chim. Acta* **1997**, *341*, 43–51.
(79) Lund-Andersen, H. *Physiol. Rev.* **1979**, *59*, 305–352.
(80) Silver, I. A. *A Microglucose Electrode* in *Ion and Enzyme Electrodes*; Kessler, M., Ed.; Munich: Ubean and Schwarzenberg, 1976, pp 189–192.
(81) Lowry, J. P.; McAteer, K.; El Atrash, S. S.; Duff, A.; O'Neill, R. D. *Anal. Chem.* **1994**, *66*, 1754–1761.
(82) Silver, I. A.; Erecinska, M. *J. Neurosci.* **1994**, *14*, 5068–5076.
(83) Netchiporouk, L. I.; Shram, N. F.; Jaffrezic-Renault, N.; Martelet, C.; Cespuglio, R. *Anal. Chem.* **1996**, *68*, 4358–4364.
(84) Dalbasti, T.; Kilinc, E.; Erdem, A. Ozsoz, M. *Biosens. Bioelectron.* **1998**, *13*, 881–888.
(85) Lowry, J. P.; O'Neill, R. D.; Boutelle, M. G.; Fillenz, M. *J. Neurochem.* **1998**, *70*, 391–396.
(86) Hu, Y.; Wilson, G. S. *J. Neurochem.* **1997**, *68*, 1745–1752.
(87) Oldendorf, W. H.; Pardridge, W. M.; Braun, L. D.; Crane, P. D. *J. Neurochem.* **1982**, *38*, 1413–1418.
(88) Crane, P. D.; Pardridge, W. M.; Braun, L. D.; Olendorf, W. H. *J. Cereb. Blood Flow Metab.* **1985**, *5*, 40–46.
(89) Mason, G. F.; Behar, K. L.; Rothman, D. L.; Shulman, R. G. *J. Cereb. Blood Flow Metab.* **1992**, *12*, 448–455.

(90) Fellows, L. K.; Boutelle, M. G.; Fillenz, M. *J. Neurochem.* **1992**, *59*, 2141−2147.
(91) Hu, Y.; Wilson, G. S. *J. Neurochem.* **1997**, *69*, 1484−1490.
(92) Shram, N. F.; Netchiporouk, L. I.; Martelet, C.; Jaffrezic-Renault, N.; Bonnet, C.; Cespuglio, R. *Anal. Chem.* **1998**, *70*, 2618−2622.
(93) Tsacopoulos, M.; Magistretti, P. J. *J. Neurosci.* **1996**, *16*, 877−885.
(94) Pellerin, L.; Magistretti, P. J. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 10625−10629.
(95) Poitry-Yamate, C. L.; Poitry, S.; Tsacopoulos M. *J. Neurosci.* **1995**, *15*, 5179−5191.
(96) Xin, Q.; Wightman, R. M. *Anal. Chem.* **1998**, *70*, 1677−1681.
(97) Pihel, K.; Hsieh, S.; Jorgenson, J. G.; Wightman, R. M. *Anal. Chem.* **1995**, *67*, 4514−4521.
(98) Rosenzweig, Z.; Kopelman, R. *Anal. Chem.* **1995**, *67*, 2650−2654.
(99) Bronk, K. S.; Michael, K. L.; Pantano, P.; Walt, D. R. *Anal. Chem.* **1995**, *67*, 2750−2757.
(100) Black, J. *Biological Performance of Materials: Fundamentals of Biocompatibility,* 2nd ed.; Marcel Dekker: New York, 1992.
(101) Shichiri, M.; Sakakida, M.; Nishida, K.; Shimoda, S. *Artif. Organs* **1998**, *22*, 32−42.
(102) Quinn, C. A. P.; Connor, R. E.; Heller, A. *Biomaterials* **1997**, *18*, 1665−1670. Quinn, C. P.; Pathak, C. P.; Heller, A.; Hubbell, J. A. *Biomaterials* **1995**, *16*, 389−396.
(103) McAuslan, B. R.; Johnson, G.; Hannan, G. N.; Norris, W. D. *J. Biomed. Mater. Res.* **1988**, *22*, 963−976.
(104) Absolom, D.; Thompson, C.; Hawthorn, L.; Zingg, W.; Neumann, A. *J. Biomed. Mater. Res.* **1988**, *22*, 215−229.
(105) Maroudas, N. *J. Theor. Biol.* **1975**, *49*, 417−424.
(106) Lewandowska, K.; Balachander, N.; Sukenik, C.; Culp, L. *J. Cell Physiol.* **1989**, *141*, 334−345.
(107) Johnson, S. D.; Anderson, J. M.; Marchant, R. E. *J. Biomed. Mater. Res.* **1992**, *26*, 915−935.
(108) Miller, K. M.; Anderson, J. M. *J. Biomed. Mater. Res.* **1989**, *22*, 713−731.
(109) *Handbook of Biomaterials Evaluation: Scientific, Technical, and Clinical Testing of Implant Materials,* 2nd ed.; Von Recum, A., Ed.; Hemisphere Publications: 1998.
(110) (a) Lindner, E.; Cosofret, V. V.; Ufer, S.; Buck, R. P.; Kao, W. J.; Neuman, M. R.; Anderson, J. M. *J. Biomed. Mater. Res. 28*, **1994**, 591−601. (b) Kao, W. J.; McNally, A. K.; Hiltner, A.; Anderson, J. M. *J. Biomed. Mater. Res.* **1995**, *29*, 1267−1275. (c) Lindner, E.; Cosofret, V. V.; Buck, R. P.; Johnson, T. A.; Ash, R. B.; Neuman, M. R.; Kao, W. J.; Anderson, J. M. *Electroanalysis* **1995**, *7*, 864−870. (d) Mathur, A. B.; Collier, T. O.; Kao, W. J.; Wiggins, M.; Schubert, M. A.; Hiltner, A.; Anderson, J. M. *J. Biomed. Mater. Res.* **1997**, *36*, 246−257.
(111) Wientjes, K. J.; Vonk, P.; Vonk-van Klei, Y.; Schoonen, A. J. M., Kossen, N. W. *Diabetes Care* **1998**, *21*, 1481−1488.
(112) Zhao, Q. H.; McNally, A. K.; Rubin, K. R.; Renier, M.; Wu, Y.; Rose-Caprara, Anderson, J. M.; Hiltner, A.; Urbanski, P.; Stokes, K. *J. Biomed. Mater. Res.* **1993**, *27*, 379−389.
(113) Sharkawy, A. A.; Klitzman, B.; Truskey, G. A.; Reichert, W. M. *J. Biomed. Mater. Res.* **1997**, *37*, 401−412. Sharkawy, A. A.; Klitzman, B.; Truskey, G. A.; Reichert, W. M. *J. Biomed. Mater. Res.* **1998**, *40*, 586−597. Sharkawy, A. A.; Klitzman, B.; Truskey, G. A.; Reichert, W. M. *J. Biomed. Mater. Res.* **1998**, *40*, 598−605.
(114) Gasiorowski, K.; Brokos, J. B.; Szyba, K.; Wozniak, D.; Fraser, D. M.; Zakeeruddin, S. M.; Graetzel, M. *Biometals* **1999**, *12*, 19−26.

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