Electrochemical Glucose Biosensors

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Contents

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1. Introduction

Diabetes mellitus is a worldwide public health problem. This metabolic disorder results from insulin deficiency and hyperglycemia and is reflected by blood glucose concentrations higher or lower than the normal range of 80−120 mg/dL (4.4−6.6 mM). The disease is one of the leading causes of death and disability in the world. The complications of battling diabetes are numerous, including higher risks of heart disease, kidney failure, or blindness. Such complications can be greatly reduced through stringent personal control of blood glucose. The diagnosis and management of diabetes mellitus thus requires a tight monitoring of blood glucose levels. Accordingly, millions of diabetics test their blood glucose levels daily, making glucose the most commonly tested analyte. Indeed, glucose biosensors account for about 85% of the entire biosensor market. Such huge market size makes diabetes a model disease for developing new biosensing concepts. The tremendous economic prospects associated with the management of diabetes along with the challenge of providing such reliable and tight glycemic control have thus led to a considerable amount of fascinating research and innovative detection strategies.[1,2] Amperometric enzyme electrodes, based on glucose oxidase (GOx), have played a leading role in the move to simple easy-to-use blood sugar testing and are expected to play a similar role in the move toward continuous glucose monitoring.

Since Clark and Lyons proposed in 1962 the initial concept of glucose enzyme electrodes,[3] we have witnessed tremendous effort directed toward the development of reliable devices for diabetes control. Different approaches have been explored in the operation of glucose enzyme electrodes. In addition to diabetes control, such devices offer great promise for other important applications, ranging from bioprocess monitoring to food analysis. The great importance of glucose has generated an enormous number of publications, the flow of which shows no sign of diminishing. Yet, in spite of the many impressive advances in the design and use of glucose biosensors, the promise of tight diabetes management has

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not been fulfilled. There are still major challenges in achieving clinically accurate continuous glycemic monitoring in connection to closed-loop systems aimed at optimal insulin delivery. Such feedback response to changes in the body chemistry has broader implications upon the management of different diseases. The management of diabetes thus represents the first example of individualized (personalized) medicine.

This review discusses the principles of operation of electrochemical glucose biosensors, examines their history, discusses recent developments and current status, surveys major strategies for enhancing their performance, and outlines key challenges and opportunities in their further development and use. Emphasis is given to fundamental advances of glucose sensing principles and related materials. It is not a comprehensive review but rather discusses key developments and applications. Given the very broad field and long history of electrochemical glucose biosensors, the author apologizes for possible oversights of important contributions.

2. Brief History of Electrochemical Glucose Biosensors

The history of glucose enzyme electrodes began in 1962 with the development of the first device by Clark and Lyons of the Cincinnati Children's Hospital.[3] Their first glucose enzyme electrode relied on a thin layer of GOx entrapped over an oxygen electrode via a semipermeable dialysis membrane. Measurements were made based on the monitoring of the oxygen consumed by the enzyme-catalyzed reaction

$$\text{glucose} + O_2 \xrightarrow{\text{glusoce oxidase}} \text{gluconic acid} + H_2O_2 \quad (1)$$

A negative potential was applied to the platinum cathode for a reductive detection of the oxygen consumption

$$O_2 + 4H^+ + 4\,e^- \rightarrow 2H_2O \quad (2)$$

The entire field of biosensors can trace its origin to this original glucose enzyme electrode. Clark's original patent[4] covers the use of one or more enzymes for converting electroinactive substrates to electroactive products. The effect of interference was corrected by using two electrodes, one of which was covered with the enzyme, and measuring the differential current. Clark's technology was subsequently transferred to Yellow Spring Instrument (YSI) Company, which launched in 1975 the first dedicated glucose analyzer (the Model 23 YSI analyzer) for direct measurement of glucose in 25 μL whole blood samples. Updike and Hicks[5] further developed this principle by using two oxygen working electrodes (one covered with the enzyme) and measuring the differential current in order to correct for the oxygen background variation in samples. In 1973, Guilbault and Lubrano[6] described an enzyme electrode for the measurement of blood glucose based on amperometric (anodic) monitoring of the hydrogen peroxide product

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (3)$$

The resulting biosensor offered good accuracy and precision in connection with 100 μL blood samples. A wide range of amperometric enzyme electrodes, differing in electrode design or material, immobilization approach, or membrane composition, has since been described. Use of electron acceptors for replacing oxygen in GOx-based blood glucose measurements was demonstrated in 1974.[7] Continuous ex-vivo monitoring of blood glucose was also proposed in 1974,[8] while in-vivo glucose monitoring was demonstrated by Shichiri et al. in 1982.[9]

During the 1980s biosensors became a 'hot' topic, reflecting a growing emphasis on biotechnology. Considerable efforts during this decade focused on the development of mediator-based 'second-generation' glucose biosensors,[10−12] introduction of commercial screen-printed strips for self-monitoring of blood glucose,[13,14] and use of modified electrodes and tailored membranes/coatings for enhancing sensor performance.[15] In the 1990s, we witnessed extensive activity directed toward the establishment of electrical communication between the redox center of GOx and the electrode surface.[16−20] Of particular note is the work of Heller, who introduced the use of flexible polymer with osmium redox sites.[16,17] During this period, we also witnessed the development of minimally invasive subcutaneously implantable devices.[1,21−24]

It is possible also to use glucose dehydrogenase (GDH) instead of GOx for amperometric biosensing of glucose. However, the construction of glucose biosensors based on GDH requires a source of NAD^+ and a redox mediator to lower the overvoltage for oxidation of the NADH product. Quinoprotein GDH can also be used in connection to a pyrroloquinoline quinone (PQQ) cofactor

$$\text{glucose} + \text{PQQ(ox)} \rightarrow \text{gluconolactone} + \text{PQQ(red)} \quad (4)$$

While eliminating the need for a NAD^+ cofactor, such PQQ enzymes have not been widely used owing to their limited stability.

3. First-Generation Glucose Biosensors

First-generation glucose biosensors rely on the use of the natural oxygen cosubstrate and generation and detection of hydrogen peroxide (eqs 1 and 3). The biocatalytic reaction involves reduction of the flavin group (FAD) in the enzyme by reaction with glucose to give the reduced form of the enzyme ($FADH_2$)

$$\text{GOx(FAD)} + \text{glucose} \rightarrow \text{GOx(FADH}_2\text{)} + \text{gluconolactone} \quad (5)$$

followed by reoxidation of the flavin by molecular oxygen to regenerate the oxidized form of the enzyme GOx(FAD)

$$\text{GOx(FADH}_2\text{)} + O_2 \rightarrow \text{GOx(FAD)} + H_2O_2 \quad (6)$$

Measurements of peroxide formation have the advantage of being simpler, especially when miniaturized devices are concerned. Such measurements are commonly carried out on a platinum electrode at a moderate anodic potential of around +0.6 V (vs Ag/AgCl). A very common configuration is the YSI probe, which involves the entrapment of GOx between an inner anti-interference cellulose acetate membrane and an outer diffusion-limiting/biocompatible one.

3.1. Electroactive Interferences

The amperometric (anodic) measurement of hydrogen peroxide at common working electrodes requires application of a relatively high potential at which endogenous reducing species, such as ascorbic and uric acids and some drugs (e.g.,

acetaminophen), are also electroactive. The current contributions of these and other oxidizable constituents of biological fluids can compromise the selectivity and hence the overall accuracy of measurement. Considerable efforts during the late 1980s were devoted to minimizing the interference of coexisting electroactive compounds.

One useful avenue in diminishing electroactive interferences is to employ a permselective coating that minimizes the access of these constituents toward the electrode surface. Different polymers, multilayers, and mixed layers with transport properties based on charge, size, or polarity have thus been used for blocking coexisting electroactive compounds.[25−31] Such films also exclude surface-active macromolecules, hence protecting the surface and imparting higher stability. Electropolymerized films, particularly poly(phenylendiamine), polyphenol, and overoxidized polypyrrole, have been shown to be extremely useful in imparting high selectivity (by rejecting interferences based on size exclusion) while confining GOx onto the surface.[25,27,28] The electropolymerization process makes it possible to generate coatings on extremely small surfaces of complex geometries, although the resulting films often have limited stability for in-vivo work. Other commonly used coatings include size-exclusion cellulose acetate films,[29] the negatively charged (sulfonated) Nafion or Kodak AQ ionomers,[30] and hydrophobic alkanethiol or lipid layers.[31] Use of overlaid multilayers, which combines the properties of different films, offers additional advantages. For example, alternate deposition of Nafion and cellulose acetate has been used to eliminate the interference of the neutral acetaminophen and negatively charged ascorbic and uric acids, respectively.[32]

Another avenue for achieving high selectivity involves the preferential electrocatalytic detection of the generated hydrogen peroxide.[33−41] Such detection relies on tuning the operating potential to the optimal region (+0.0 to −0.20 V vs Ag/AgCl) where contributions from easily oxidizable interfering substances are eliminated. Remarkably high selectivity coupled with a fast and sensitive response has thus been obtained. For example, a substantial lowering of the overvoltage for the hydrogen peroxide redox process, and hence a highly selective glucose sensing, can be achieved using metal−hexacyanoferrate-based transducers.[36−41] In particular, Prussian-Blue (PB; ferric−ferrocyanide) modified electrodes have received considerable attention owing to their very strong and stable electrocatalytic activity. Karyakin et al. showed the catalytic rate constant for H_2O_2 reduction at PB film to be 3×10^3 M^{-1} s^{-1}.[38] Prussian-Blue offers a substantial lowering of the overvoltage for the hydrogen peroxide redox process and hence permits highly selective biosensing of glucose at a very low potential (−0.1 V vs Ag/AgCl). The high catalytic activity of PB leads also to a very high sensitivity toward hydrogen peroxide. Further improvements in the stability and selectivity of PB-based hydrogen peroxide transducers can be obtained by electropolymerizing a nonconducting poly(1,2-diaminobenzne) permselective coating on top of the PB layer.[39] A glucose nanosensor, based on the co-deposition of PB and GOx on a carbon-fiber nanoelectrode, has also been reported.[40] PB-based carbon inks were developed for fabricating electrocatalytic screen-printed glucose biosensors.[41]

Similarly, metallized carbons such as rhodium or ruthenium on carbon[33−35] have been shown to be extremely useful for highly selective biosensing of glucose. The high selectivity of metallized carbon transducers (such as Rh−C or Ru−C) reflects their strong preferential electrocatalytic detection of hydrogen peroxide at an optimal potential range around 0.0 V, where most unwanted background reactions are negligible. Such catalytic oxidation of the peroxide product relies on the presence of a metal oxide film. The hydrogen peroxide reduces the surface metal oxide film to the metal, which is then reoxidized electrochemically, generating the anodic current signal. Miniaturized or disposable glucose microsensors have thus been prepared by electrochemical co-deposition of ruthenium and glucose oxidase onto carbon fiber microelectrodes[35] or dispersing metal microparticles or metallized carbon particles within screen-printable inks.[33,34] Additional improvements can be achieved by combining this preferential catalytic activity with a discriminative layer, e.g., by dispersing rhodium particles within a Nafion film.[42] Low-potential selective detection of the GOx-generated hydrogen peroxide is possible also by coupling with another enzyme horseradish peroxidase (HRP) that catalyzes the peroxide oxidation.[45] The marked reduction in the overvoltage for hydrogen peroxide at carbon-nanotube (CNT)-modified electrodes offers highly selective low-potential biosensing of glucose.[43,44] Yet, some controversy exists on whether the improved electrochemical behavior of hydrogen peroxide at CNT electrodes reflects the intrinsic CNT electrocatalysis or associated with metal impurities. Low-potential selective detection of the GOx-generated hydrogen peroxide is possible also by coupling with another enzyme such as horseradish peroxidase (HRP) that catalyzes the peroxide oxidation.[45] The coupling of CNT with platinum nanoparticles has been shown to be extremely useful for enhancing the sensitivity and speed of GOx-based glucose biosensors (down to 0.5 μM within 3 s).[46] Use of CNT molecular wires, connecting the electrode and the redox center of GOx, will be discussed in section 4.5.

3.2. Oxygen Dependence

Since oxidase-based devices rely on the use of oxygen as the physiological electron acceptor, they are subject to errors resulting from fluctuations in oxygen tension and the stoichiometric limitation of oxygen. These errors include changes in sensor response and a reduced upper limit of linearity. This limitation (known as the "oxygen deficit") reflects the fact that normal oxygen concentrations are about 1 order of magnitude lower than the physiological level of glucose.

Several avenues have been proposed for addressing this oxygen limitation. One approach relies on the use of mass-transport-limiting films (such as polyurethane or polycarbonate) for tailoring the flux of glucose and oxygen, i.e., increasing the oxygen/glucose permeability ratio.[1,47,48] A two-dimensional cylindrical electrode, designed by Gough's group,[47,48] has been particularly attractive for addressing the oxygen deficit by allowing oxygen to diffuse into the enzyme region of the sensor from both directions while glucose diffuses only from one direction (of the exposed end). This was accomplished by using a two-dimensional sensor design with a cylindrical gel containing GOx and an outside silicone rubber tube which is impermeable to glucose but highly permeable to oxygen. We addressed the oxygen limitation of glucose biosensors by designing oxygen-rich carbon paste enzyme electrodes.[49,50] This biosensor is based on a fluorocarbon (Kel-F oil) pasting liquid, which has very high oxygen solubility, allowing it to act as an internal source of oxygen. The internal flux of oxygen can thus support the enzymatic

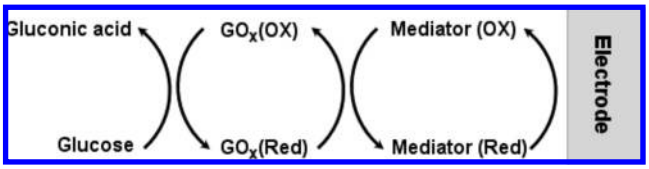

Figure 1. Sequence of events that occur in 'second-generation' (mediator-based) glucose biosensors-mediated system.

reaction, even in oxygen-free glucose solutions. It is possible also to circumvent the oxygen demand issue by replacing the GOx with glucose dehydrogenase (GDH), which does not require an oxygen cofactor.[51]

4. Second-Generation Glucose Biosensors

4.1. Electron Transfer between GOx and Electrode Surfaces

Further improvements (and solutions to the above errors) can be obtained by replacing the oxygen with a nonphysiological (synthetic) electron acceptor capable of shuttling electrons from the redox center of the enzyme to the surface of the electrode. The transfer of electrons between the GOx active site and the electrode surface is the limiting factor in the operation of amperometric glucose biosensors. Glucose oxidase does not directly transfer electrons to conventional electrodes because of a thick protein layer surrounding its flavin adenine dinucleotide (FAD) redox center and introducing an intrinsic barrier to direct electron transfer. Accordingly, different innovative strategies have been suggested for establishing and tailoring the electrical contact between the redox center of GOx and electrode surfaces.[52−54]

4.2. Use of Nonphysiological Electron Acceptors

Particularly useful in developing glucose biosensors has been the use of artificial mediators that shuttle (carry) electrons between the FAD center and the electrode surface by the following scheme

$$\text{glucose} + \text{GOx}_{(\text{ox})} \rightarrow \text{gluconic acid} + \text{GOx}_{(\text{red})} \quad (7)$$

$$\text{GOx}_{(\text{red})} + 2\text{M}_{(\text{ox})} \rightarrow \text{GOx}_{(\text{ox})} + 2\text{M}_{(\text{red})} + 2\text{H}^+ \quad (8)$$

$$2\text{M}_{(\text{red})} \rightarrow 2\text{M}_{(\text{ox})} + 2\text{e}^- \quad (9)$$

where $M_{(ox)}$ and $M_{(red)}$ are the oxidized and reduced forms of the mediator. The reduced form is reoxidized at the electrode, giving a current signal (proportional to the glucose concentration) while regenerating the oxidized form of the mediator (eq 9). Such mediation cycle is displayed in Figure 1.

Diffusional electron mediators, such as ferrocene derivatives, ferricyanide, conducting organic salts (particularly tetrathiafulvalene-tetracyanoquinodimethane, TTF-TCNQ), quinone compounds, transition-metal complexes, and phenothiazine and phenoxazine compounds, have been particularly useful to electrically contact GOx.[9−12] The former received considerable attention owing to their low (pH-independent) redox potentials and larger number of derivatives. As a result of using these electron-carrying mediators, measurements become largely independent of oxygen partial pressure and can be carried out at lower potentials that do not provoke interfering reactions from coexisting electroactive species. In order to function effectively, the mediator should react

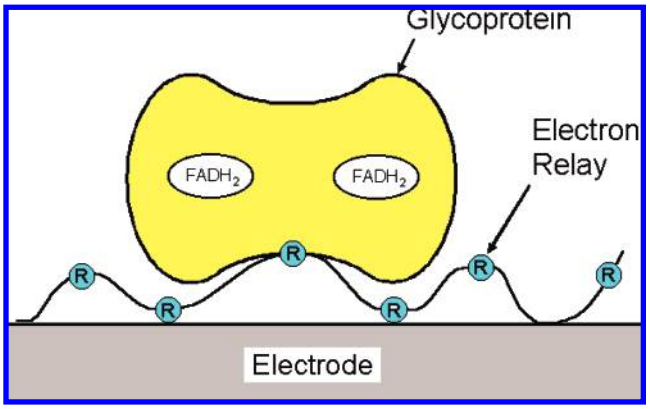

Figure 2. Use of a redox polymer for wiring GOx: efficient electrical communication between the redox center of the enzyme and electrode surfaces.

rapidly with the reduced enzyme (to minimize competition with oxygen), possess good electrochemical properties (such as a low pH-independent redox potential), and have low solubility in aqueous medium. The mediator must also be insoluble, nontoxic, and chemically stable (in both reduced and oxidized forms). The oxygen competition can be minimized if the rate of electron transfer via the mediator is high compared to the rate of the enzyme reaction with oxygen. In most cases, however, oxidation of the reduced GOx by oxygen can occur even in the presence of mediator (particularly as oxygen is freely diffusing), hence limiting the accuracy (especially at low glucose levels). In addition, the low potential of most mediators minimizes but does not eliminate the oxidation of endogenous species (particularly ascorbate). Such endogenous electroactive compounds can also consume the mediator, leading to additional errors. Commercial blood glucose self-testing meters, described in section 7, commonly rely on the use of ferricyanide or ferrocene mediators. Most in-vivo devices, however, are mediatorless due to potential leaching and toxicity of the mediator. Mediated systems also display low stability upon an extended continuous operation.

4.3. Wired Enzyme Electrodes

Enzyme wiring with a redox polymer offers additional improvements in the electrical contact between the redox center of GOx and electrode surfaces (Figure 2). An elegant nondiffusional route for establishing a communication link between GOx and electrodes was developed by Heller's group.[16,55] This was accomplished by 'wiring' the enzyme to the surface with a long flexible hydrophilic polymer backbone [poly(vinylpyridine) or poly(vinylimidazole)] having a dense array of covalently linked osmium-complex electron relays. The redox polymer penetrates and binds the enzyme (through multiple lysine amines) to form a three-dimensional network that adheres to the surface. Such folding along the GOx dramatically reduces the distance between the redox centers of the polymer and the FAD center of the enzyme. The resulting film conducts electrons and is permeable to the substrate and product of the enzymatic reaction. Electrons originating from the redox site of GOx are thus transferred through the gel's polymer network to the electrode. The resulting three-dimensional redox-polymer/enzyme networks thus offer high current outputs and fast response and stabilize the mediator to electrode surfaces. Current densities as high as mA/cm^2 were reached upon wiring multiple enzyme layers. Such huge current densities

facilitate the use of ultrasmall enzyme electrodes. The remarkable sensitivity is coupled with very high selectivity (e.g., negligible interferences from ascorbic and uric acids, acetaminophen, and cysteine at +0.20V vs SCE).[56] Such wired enzyme electrodes are particularly attractive for in-vivo applications where leaching of diffusional mediators is to be avoided and when small size is important.

4.4. Modification of GOx with Electron Relays

Chemical modification of GOx with electron-relay groups represents another attractive route for facilitating the electron transfer between the GOx redox center and the electrode surface. In 1984 Hill described the covalent attachment of ferrocene−monocarboxylic acid to the lysine residues of GOx using isobutyl choloformate,[11] while Heller[16] used carbodimide coupling for attaching the same mediator to GOx. Such covalent attachment of ferrocene groups led to direct oxidation of the flavin center of GOx at unmodified electrodes with the bound ferrocenes allowing electron tunneling in a number of consecutive steps. Bartlett described the carbodimide-based covalent attachment of TTF to the peptide backbone of GOx.[20] Direct oxidation of the FAD centers of the enzyme was demonstrated without the need for soluble species.

Glucose biosensors with extremely efficient electrical communication with the electrode can be generated by the enzyme reconstitution process. Willner's group[57] reported on an elegant approach for modifying GOx with electron relays and obtaining efficient electrical contact. For this purpose, the FAD active center of the enzyme was removed to allow positioning of an electron-mediating ferrocene unit prior to the reconstitution of the apoenzyme with the modified FAD. The attachment of electron-transfer relays at the enzyme periphery has also been considered by the same group for yielding short electron-transfer distances.[52,54] While clearly illustrating a direct coupling, demonstration of a stable response would be required prior to practical applications of this elegant approach.

4.5. Nanomaterial Electrical Connectors

The emergence of nanotechnology has opened new horizons for the application of nanomaterials in bioanalytical chemistry. Recent advances in nanotechnology offer exciting prospects in the field of bioelectronics. Owing to the similar dimensions of nanoparticles and redox proteins such nanomaterials can be used for effective electrical wiring of redox enzymes. Various nanomaterials, including gold nanoparticles or carbon nanotubes (CNT), have thus been used as electrical connectors between the electrode and the redox center of GOx. For example, apo-glucose oxidase can be reconstituted on a 1.4 nm gold nanocrystal functionalized with the FAD cofactor.[58] The gold nanoparticle, immobilized onto the gold electrode by means of a dithiol linker, thus acts as an "electrical nanoplug" (relay unit) for the electrical wiring of its redox-active center. This leads to a high electron-transfer turnover rate of ~5000 per second. Carbon nanotubes (CNT) represent additional nanomaterials that can be coupled to enzymes to provide a favorable surface orientation and act as an electrical connector between their redox center and the electrode surface. Particularly useful for this task have been vertically aligned CNTs that act as molecular wires ('nanoconnectors') between the underlying electrode and a redox enzyme.[59−61] Willner's group[59] demonstrated that aligned reconstituted glucose oxidase (GOx) on the edge of single-wall carbon nanotubes (SWCNT) can be linked to an electrode surface (Figure 3). Such enzyme reconstitution on the end of CNT represents an extremely efficient approach for 'plugging' an electrode into GOx. Electrons were thus transported along distances higher than 150 nm with the length of the SWCNT controlling the rate of electron transport. An interfacial electron-transfer rate constant of 42 s^{-1} was estimated for 50 nm long SWCNT. Efficient direct electrical connection to GOx was reported also by Gooding's group in connection to aligned SWCNT arrays.[60] At present, activation of the bioelectrocatalytic functions of GOx by nanoparticles or CNT requires electrical overpotentials (beyond the thermodynamic redox potential of the enzyme redox center). Improving the contact between the nanomaterial and the electrode might decrease this overpotential.

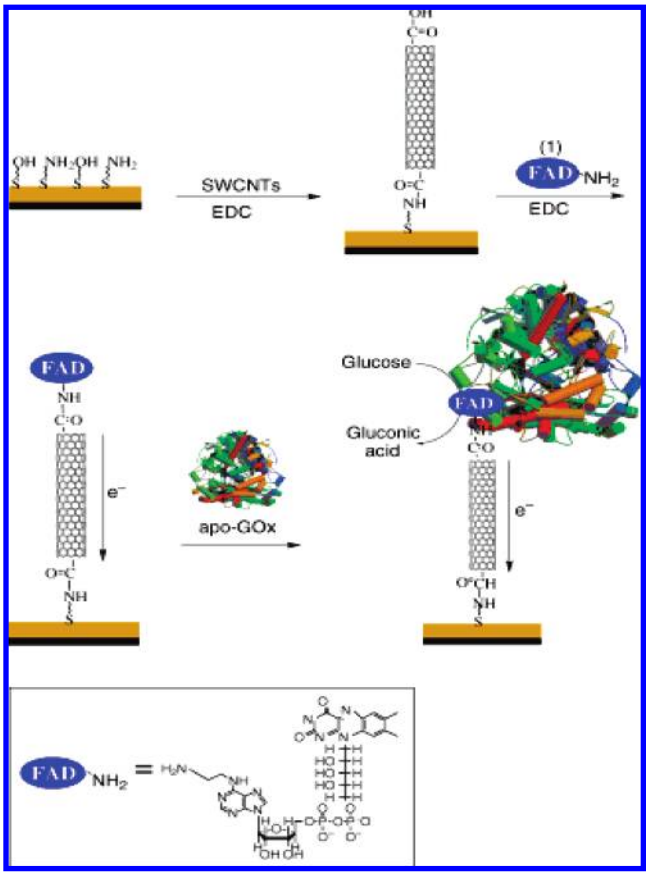

Figure 3. Carbon nanotube (CNT) connectors with long-range electrical contacting. Assembly of the CNT electrically contacted glucose oxidase electrode. (Reprinted with permission from ref 59. Copyright 2004 Wiley-VCH.)

5. Toward Third-Generation Glucose Biosensors

Ultimately, one would like to eliminate the mediator and develop a reagentless glucose biosensor with a low operating potential, close to that of the redox potential of the enzyme. In this case, the electron is transferred directly from glucose to the electrode via the active site of the enzyme. The absence of mediators is the main advantage of such *third-generation biosensors*, leading to a very high selectivity (owing to the very low operating potential). However, as discussed earlier, critical challenges must be overcome for the successful realization of this direct electron-transfer route owing to the spatial separation of the donor−acceptor pair. Efficient direct

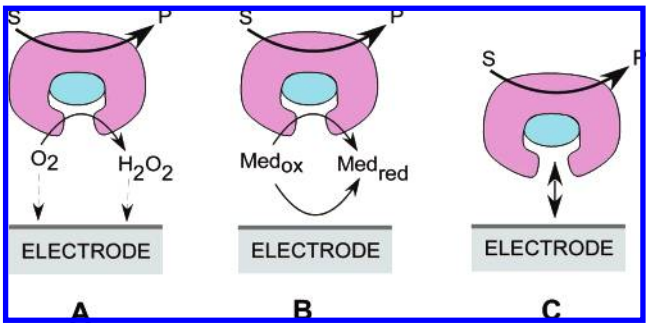

Figure 4. Three generations of amperometric enzyme electrodes for glucose based on the use of natural oxygen cofactor (A), artificial redox mediators (B), or direct electron transfer between GOx and the electrode (C).

electron transfer at conventional electrodes has been reported only for few redox enzymes. There are mixed reports in the literature regarding the direct (mediatorless) electron transfer catalyzed by GOx.[53] Although several papers claim such direct electron transfer between GOx and the electrode, only few provide the level of proof for such mediatorless detection. Unsuccessful efforts to obtain direct electron transfer of GOx at conventional electrodes led to exploration of new electrode materials. The optimally designed electrode configuration has to ensure that the electron-transfer distance between the immobilized protein and the surface is made as short as possible. One route for creating third-generation amperometric glucose biosensors is to use conducting organic salt electrodes based on charge-transfer complexes such as tetrathiafulvalene-tetracyanoquinodimethane (TTF-TCNQ).[62−64] Different electron-transfer mechanisms at TTF-TCNQ electrodes have been proposed by different authors, and the precise mechanism of GOx catalysis remains controversial. Khan et al.[63] described a third-generation amperometric glucose sensor based on a stable charge-transfer complex electrode. The device relied on the growing tree-shaped crystal structure of TTF-TCNQ. The authors claimed that the close proximity and favorable orientation of the enzyme at the crystal surface allowed direct oxidation of the enzyme and selective glucose measurements at 0.1 V (vs Ag/AgCl), although they did not provide a convincing evidence for such direct electron transfer. Palmisano et al.[64] described a disposable third-generation amperometric glucose sensor based on growing TTF-TCNQ tree-like crystals through an anti-interference layer of a nonconducting polypyrrole film. Arguments against direct electron transfer were presented by Cenas and Kulys.[65] These authors suggested that the electron transfer of GOx at TTF-TCNQ electrodes is mediated and involves corrosion of the TTF-TCNQ to produce dissolved components of these organic salts that mediate the electron transfer of the enzyme. Mediatorless third-generation glucose biosensors based on the GOx/polypyrrole system were suggested by Aizawa[66] and Koopal.[67] However, the relatively high anodic potential of this system (vs the redox potential of $FAD/FADH_2$, −0.44 V) suggests the possibility of electron transfer mediated by oligomeric pyrroles present on the surface. Oxidized boron-doped diamond electrodes also indicated recently some promise for mediator-free glucose detection based on direct electron transfer.[68]

Figure 4 summarizes various generations of amperometric glucose biosensors based on different mechanisms of electron transfer, including the use of natural secondary substrates, artificial redox mediators, or direct electron transfer. Although substantial progress has been made on the electronic coupling of GOx, further improvements in the charge transport between its FAD redox center and electrodes are desired.

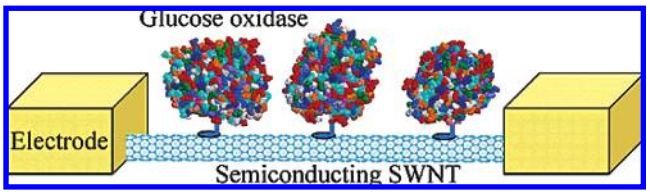

Figure 5. Carbon nanotube (CNT)-based transistor for biosensing of glucose. Schematic picture of two electrodes connecting a semiconducting CNT with GOx enzymes immobilized on its surface. (Reprinted with permission from ref 69. Copyright 2003 American Chemical Society.)

6. Solid-State Glucose Sensing Devices

The unique electrical properties of 1-dimensional nanomaterials, such as carbon nanotubes, have been shown to be useful for developing conductivity based nanosensors for glucose.[69] Dekker's group demonstrated that GOx-coated semiconducting SWNTs act as sensitive pH sensors and that the conductance of GOx-coated semiconducting SWNTs changes upon addition of glucose substrate (Figure 5). A conductivity-based glucose nanobiosensor based on conducting-polymer-based nanogap has been developed by Tao and co-workers.[70] Such nanojunction-based sensor was formed by using polyaniline/glucose oxidase for bridging a pair of nanoelectrodes separated with a small gap (ca. 20−60 nm).

Solid-state transistor-like switchable glucose sensing devices were reported earlier by Bartlett's group.[71] Such 'enzyme-transistor' responsive to glucose was prepared by connecting two carbon band microelectrodes with poly(aniline) (PANI) film covered with a GOx/poly(1,2-diaminobenzene) layer. Addition of glucose, in the presence of TTF^+, resulted in a conductivity change associated with the reduction of poly(aniline) by the enzyme mediated by TTF^+

$$TTF + PANI(ox) \rightarrow TTF^+ + PANI(red) \qquad (10)$$

7. Home Testing of Blood Glucose

Electrochemical biosensors are well suited for addressing the needs of personal (home) glucose testing and have played a key role in the move to simple one-step blood sugar testing. Since blood glucose home testing devices are used daily to diagnose potentially life-threatening events they must be of extremely high quality. The majority of personal blood glucose monitors rely on disposable screen-printed enzyme electrode test strips.[72,73] Such single-use electrode strips are mass produced by the rapid and simple thick-film (screen-printing) microfabrication or vapor deposition process.[34,74] The screen-printing technology involves printing patterns of conductors and insulators onto the surface of planar solid (plastic or ceramic) substrates based on pressing the corresponding inks through a patterned mask. Each strip contains the printed working and reference electrodes, with the working one coated with the necessary reagents (i.e., enzyme, mediator, stabilizer, surfactant, linking, and binding agents) and membranes (Figure 6). The reagents are commonly dispensed by an ink-jet printing technology and deposited in the dry form. A counter electrode and an additional ('baseline') working electrode may also be included. Various membranes (mesh, filter) are often incorporated into the test strips and along with surfactants are used to provide a

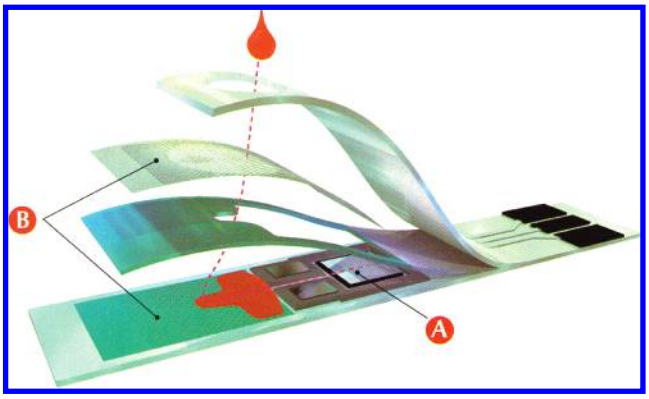

Figure 6. Cross section of a commercial strip for self-testing of blood glucose (based on the Precision biosensor manufactured by Abbott Inc.): (A) electrode system; (B) hydrophobic layer (drawing the blood).

uniform sample coverage and separate the blood cells. Such single-use devices eliminate problems of carry over, cross contamination, or drift. Overall, despite their low cost and mass production such sensor strips are based on a high degree of sophistication essential for ensuring high clinical accuracy.

The control meter is typically small (pocket-size), light, and battery operated. It relies on a potential-step (chronoamperometric) operation in connection with a short incubation (reaction) step. Such devices offer considerable promise for obtaining the desired clinical information in a simpler ("user-friendly"), faster, and cheaper manner compared to traditional assays. In 1987 Medisense Inc. (in the United Kingdom) launched the first product of this type, the pen-style Exactech device, based on the use of a ferrocene-derivative mediator. Since then, over 40 different commercial strips and pocket-sized monitors have been introduced for self-testing of blood glucose.[73,75] However, over 90% of the market consists of products manufactured by four major companies, including Life Scan, Roche Diagnostics, Abbott, and Bayer. Most of these meters rely on a ferricyanide mediator, except for the Abbott devices that employ a ferrocene derivative or an osmium-based redox polymer. In all cases, the diabetic patient pricks the finger, places the small blood droplet on the sensor strip, and obtains the blood glucose concentration (on a LC display) within 5−30 s. Some of the new meters allow sampling of submicroliter blood samples from the forearm, thus reducing the pain and discomfort associated with piercing the skin. For example, the FreeStyle monitor of Abbott relies on coulometric strips with a 50 μm gap thin-layer cell for assays of 300 nL blood samples. Widespread use of such alternative sampling sites requires that the collected samples properly reflect the blood glucose values (especially when these levels change rapidly). In addition to fast response and small size, modern personal glucose meters have features such as extended memory capacity and computer downloading capabilities. Overall, the attractive performance of modern blood glucose monitors reflects significant technological advances along with major fundamental developments (described in previous sections). Despite these remarkable technological advances, home testing of blood glucose often suffers from low and irregular testing frequency (related to the inconvenience and discomfort), inadequate interpretation of the results by the patient, or liability issues and requires compliance by patients. More integrated devices, offering multifunctional capability, enhanced interface with the physician's work, and convenient tracking of changes in the glucose level, are expected in the near future.[73]

8. Continuous Real Time in-Vivo Monitoring

Although self-testing is considered a major advance in glucose monitoring, it is limited by the number of tests per day it permits. The inconvenience associated with standard finger-stick sampling deters patients from frequent monitoring. Such testing neglects the monitoring of nighttime variations. This means that measurements do not reflect the overall direction, trends, and patterns of the blood glucose level. Hence, they may result in poor approximation of blood glucose variations. Tighter glycemic control, through more frequent measurements or continuous monitoring, is desired for detecting sharp changes in the glucose level and triggering proper alarm in cases of hypo- and hyperglycemia. Continuous glucose monitoring provides maximal information about changing blood glucose levels throughout the day, including the direction, magnitude, duration, and frequency of such fluctuations.

Continuous glucose monitoring thus addresses the deficiencies of test-strip-based meters and provides the opportunity of making fast and optimal therapeutic interventions (i.e., insulin delivery).[76] This would minimize short-term crises and long-term complications of diabetes and lead to improved quality and length of life for people with diabetes. Glucose biosensors are thus key components of closed-loop glycemic control systems for regulating a person's blood glucose. The concept of closed-loop (sense/release) systems is expected to have a major impact upon the treatment and management of other diseases and revolutionize patient monitoring.[77,78] Such a 'sense and act' route for diabetes management system represents the first example of an individualized drug administration system for optimal therapeutic intervention. Legal and liability issues may impede the practical implementation of the 'sense and act' approach since a potential false high response from the in-vivo sensor may lead to an insulin overdose.

A wide range of possible in-vivo glucose electrochemical biosensors, based on different needle designs, materials, and membrane coatings, has been studied over the past 25 years. The first application of such devices for in-vivo glucose monitoring was demonstrated by Shichiri et al. in 1982.[9] His group's needle-type glucose sensor relied on a platinum anode held at +0.6 V (vs a silver cathode), which was used to monitor the enzymatically produced hydrogen peroxide. The enzyme (GOx) entrapment was accomplished in connection with a cellulose-diacetate/heparin/polyurethane coating. The majority of glucose sensors used for in-vivo applications are based on the GOx-catalyzed oxidation of glucose by oxygen owing to concerns about potential leaching of mediators.

8.1. Requirements

The major requirements of clinically accurate in-vivo glucose sensors have been discussed in several review articles.[1,23,76,79] The ideal sensor would be one that provides a reliable real-time continuous monitoring of all blood glucose variations throughout the day with high selectivity and speed over extended periods under harsh conditions. The challenges for meeting these demands include rejection of the sensor by the body, miniaturization, long-term stability of the enzyme and transducer, oxygen deficit, in-vivo

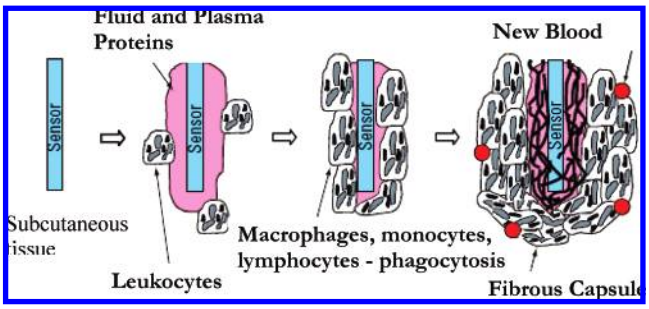

Figure 7. Inflammatory response of implantable sensor in the subcutaneous tissue. Sequence of events that leads to formation of fibrous capsules around chemical sensors. (Reprinted with permission from ref 80. Copyright 2006 American Chemical Society.)

calibration, short stabilization times, baseline drift, safety, and convenience. The sensor must be of a very tiny size and proper shape that allows for easy implantation and results in minimal discomfort. Last but not least is the powering of an autonomous sensor−transmitter system. Reducing the size of the power source (e.g., biofuel cell, battery) remains a major challenge.

Undesirable interactions between the surface of the implanted device and biological medium cause deterioration of the sensor performance upon implantation and proved to be the major barrier to the development of reliable in-vivo glucose probes. Such adverse effects include the effect of the sensor upon the host environment as well as the environment effect upon the sensor performance. In blood, the major source of complication arises from surface fouling of the electrode by proteins and coagulation composites and the risk of thromboembolism. Due to this severe blood-induced biofouling (that suppresses the glucose response), most glucose biosensors lack the biocompatibility necessary for reliable prolonged operation in whole blood. The danger of thrombus formation is another major concern (health risk) hindering the implementation of sensors implanted in the blood. Accordingly, the majority of the sensors being developed for continuous glucose monitoring do not measure blood glucose directly.

Alternative sensing sites, particularly the subcutaneous tissue, have thus received growing attention. The subcutaneous tissue is minimally invasive, and its glucose level reflects the blood glucose concentration. However, such subcutaneous implantation generates a wound site that experiences an intense local inflammatory reaction. This inflammatory response associated with the wound formation is characterized with problems such as scar tissue formation accompanied by adhesion of bacteria and macrophage and distortion of the glucose concentration in the immediate vicinity of the sensor (Figure 7). The extent of this inflammatory response depends upon various factors, including the shape, size, and rigidity of the sensor as well as its physical and chemical character.[1]

Recent approaches for designing more biocompatible in-vivo glucose sensors focused on preparing interfaces that resist biofouling. These include a controlled release of nitric oxide (NO),[80−83] protecting the outer surface with polymeric coatings (such as polyethylene glycols, polyethylene oxides, or the perfluorinated ionomer Nafion) that exhibit low protein adsorption[84−86] or co-immobilization of the anticoagulant heparin.[87] The former is attributed to the discovery that NO is an effective inhibitor of platelet and bacterial adhesion. Such NO-release glucose sensors were prepared by doping the outer polymeric membrane coating of previously reported

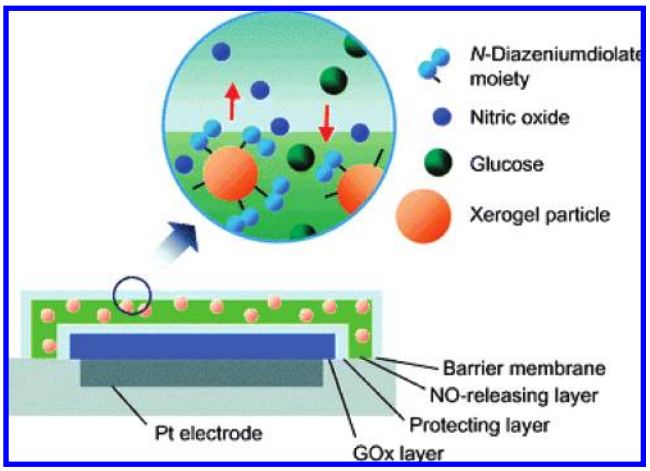

Figure 8. Nitric oxide-releasing coating for improved biocompatibility of glucose biosensors. Schematic of the hybrid xerogel/polyurethane glucose biosensor employing NO-donor-modified sol−gel particles supported in a polyurethane matrix. (Reprinted with permission from ref 81. Copyright 2004 American Chemical Society.)

needle-type electrochemical sensors with suitable lipophilic diazeniumdiolate species[82] or diazeniumdiolate-modified sol−gel particles (Figure 8).[81] Histological examination of the implant site demonstrated a significant decrease in the inflammatory response. Similarly, poly(ethylene glycol) (PEG) containing polymers are among the least protein absorbing. Quinn et al.[85] reported on a glucose permeable hydrogel based on cross-linking an 8-armed amine-terminated PEG derivative with a di-succinimidyl ester of a dipropionic acid derivative of PEG. The gel was evaluated as a biocompatible interface between an amperometric glucose microsensor and the subcutaneous tissue of a rat. Very few adherent cells were observed after 7 days.

Calibration, i.e., the transformation of the time-dependent current signal $i(t)$ into an estimation of glucose concentration at time t, $C_G(t)$, represents another major challenge to the development of sensors for continuous monitoring of glucose. This can be accomplished using one-point[88] or two-point[89] calibration procedures. In the one-point calibration procedure, the sensor sensitivity S is determined from a single blood glucose determination as the ratio between the current signal i and the blood glucose concentration C_G. Such "one-point" in-vivo calibration can be used for highly selective sensors having a zero output current at zero glucose concentration.[88] A single withdrawn blood sample can thus provide the one calibration point. If the intercept i_o is not negligible, a two-point calibration procedure is essential.[89] The two-point calibration involves an estimate of two parameters: the sensor sensitivity S and the intercept i_o (the sensor output observed in the absence of glucose). The glucose concentration at any time can thus be estimated from the current i

$$C_G(t) = (i - i_o)/S \tag{11}$$

Proper calibration thus ensures that the measured tissue glucose concentration accurately reflects the blood glucose level. A key issue is still maintaining the calibration over a period of several days. The calibration process should be repeated during implantation to account for variations in sensitivity. A calibration-free operation is the ultimate goal, but this would require detailed understanding of the sensitivity changes along with highly reproducible devices.

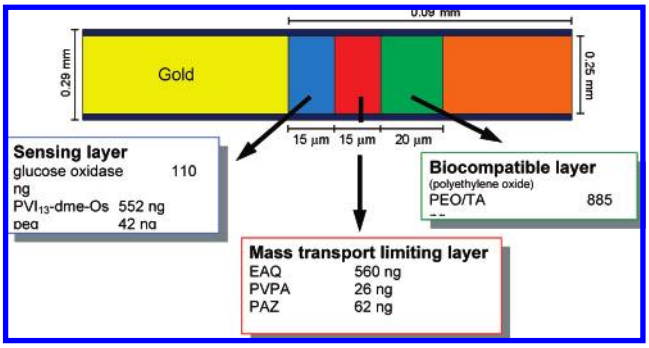

Figure 9. Design of an implantable four-layered glucose biosensor for subcutaneous monitoring. (Reprinted with permission from ref 88. Copyright 1994 American Chemical Society.)

Although major advances have been made and several short-term in-vivo glucose sensors are approaching the commercial stage, major efforts are required before a reliable long-term minimally invasive or noninvasive sensing becomes a reality.

8.2. Subcutaneous Monitoring

Most of the recent attention regarding real-time in-vivo monitoring has been given to the development of subcutaneously implantable needle-type electrodes.[21−24] Such devices track blood glucose levels by measuring the glucose concentration in the interstitial fluid of the subcutaneous tissue (assuming the ratio of the blood/tissue levels is constant). Subcutaneously implantable devices are commonly designed to operate for a few days, after which they are replaced by the patient. They are commonly inserted into the subcutaneous tissue in the abdomen or upper arm. Success in this direction has reached the level of short-term human implantation; continuously functioning devices, possessing adequate (>1 week) stability, are expected in the very near future. Such devices would enable a swift and appropriate corrective action through use of a closed-loop insulin delivery system, i.e., an artificial pancreas. Computer algorithms correcting for the transient difference (short time lag) between blood and tissue glucose concentrations have been developed.[24] These algorithms will be used in future closed-loop feedback systems to calculate the right amount of dispensed insulin.

Subcutaneously implantable glucose sensors have moved from the purely experimental stage to commercially available products.[90,91] The CGMS unit of Medtronic Minimed Inc. (Sylmar, CA) offers a 72 h period of such subcutaneous monitoring with measurement of tissue glucose every 5 min (nearly 300 readings per day) and data storage in the monitor's memory.[90] After 72 h, the sensor is removed and the information is transferred to a computer that identifies patterns of glucose variations. It was recommended that the management decision should rely on trends in the sensor recording and not upon a single-point reading.[92]A similar system is currently being developed by Abbott Inc.[91] This system is based on the wired enzyme technology of Heller's group (Figure 9), which involves insertion of a short needle into the skin and yields a reading every minute. The implanted element, designed to function for about 4 days between replacements, is small enough to be painlessly replaced by the user. Both the Abbott and Minimed devices include a limited range transmitter that relays the sensor data to a pager-like device that provides the necessary warnings and stores the data. Heller's team has engineered a miniaturized glucose−oxygen biofuel cell, based on an implantable 7 μm carbon fiber anode and cathode (coated with GOx and laccase, respectively), for powering the autonomous sensor−transmitter system.[93] Additional devices based on patch-like sensors, nanoneedles, and microdialysis sampling are currently being developed by different organizations. The later are discussed below.

8.3. Toward Noninvasive Glucose Monitoring

Noninvasive glucose sensing is the ultimate goal of glucose monitoring. This noninvasive route for continuous glucose monitoring is expected to eliminate the challenges, pain, and discomfort associated with implantable devices. Noninvasive methods are thus preferable to invasive techniques, provided that they do not compromise the clinical accuracy. Such ability to measure glucose noninvasively will thus revolutionize the treatment of diabetes. This approach has been directed toward glucose measurements in saliva, tears, or sweat. In particular, Cygnus Inc. has developed a watch-like glucose monitor based on the coupling of reverse iontophoretic collection of glucose across the skin with the biosensor function.[94] The wearable GlucoWatch device (available now from Animas Technologies Inc.) contains both the extraction and the sensing functions along with the operating and data-storage circuitry. It provides up to three glucose readings per hour for up to 12 h (i.e., 36 readings within a 12 h period). The system has been shown to be capable of measuring the electroosmotically extracted glucose with a clinically acceptable level of accuracy. An alarm capability is included to alert the individual of very low or high glucose levels. However, the unit requires a long warm up and calibration against fingerstick blood measurement and is subject to difficulties due to skin rash with irritation under the device, long warm up times, sweating, or change in the skin temperature. Other routes for "collecting" the glucose through the skin and for noninvasive glucose testing are currently being examined by various groups and companies. Most of these approaches rely on optical detection, which is beyond the scope of this review. Despite these extensive efforts, no reliable method is presently available for continuous noninvasive glucose monitoring and it is still uncertain if such reliable monitoring will become available in the near future.

8.4. Microdialysis Sampling

Another alternative to implanted needle glucose biosensors is to use microdialysis as an interface between the body and the biosensor. Here a hollow dialysis fiber is commonly implanted in the subcutaneous tissue and perfused with isotonic fluid. Glucose, diffusing from the tissue into the fiber, is thus pumped toward to the enzyme electrode. Various groups developed portable systems for continuous tissue glucose monitoring based on such combination of microdialysis and enzymatic amperometric glucose measurement.[95−100] For example, Vering et al.[96] described a microdialysis-based wearable system for continuous in-vivo monitoring of glucose. Sampling was performed by means of a biocompatible microdialysis needle probe inserted into the subcutaneous tissue. A microfabricated enzyme electrode was used in connection to a stopped-flow procedure. Langerman et al. applied a microdialysis system for determining glucose and lactate in the brain tissue of injured critical care patients.[95] Several companies, such as Menarini Diagnostics

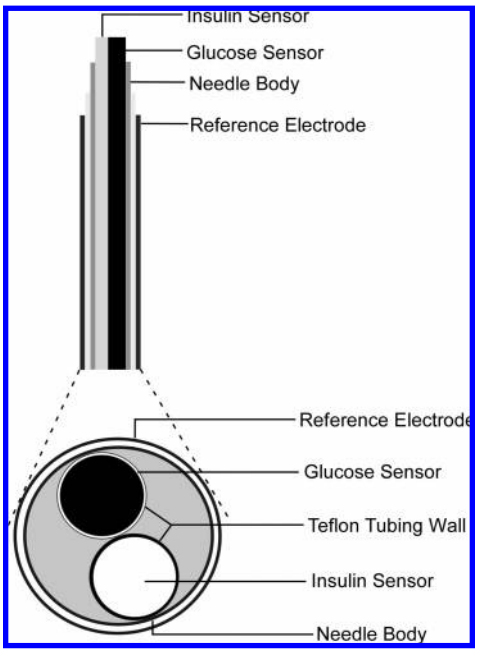

Figure 10. Integrated needle-type glucose/insulin microsensor based on electrocatalytic (RuOx) insulin detection and biocatalytic (GOx) glucose sensing. (Reprinted with permission from ref 103. Copyright 2001 American Chemical Society.)

or Roche, are currently exploring commercial microdialysis glucose monitoring probes. The GlucoDay microdialysis system of Menarini Diagnostics displayed good correlation with venous blood glucose measurements of 70 diabetes patients.[100] The Roche (Disentronic) system is non-enzymatic and relies on monitoring glucose-induced changes in the viscosity associated with binding to the lectin concanavalin A.[97]

8.5. Dual-Analyte Detection

Various clinical situations require the simultaneous monitoring of glucose and of other clinically important analytes, such as lactate or insulin. Such coupling of two sensing elements requires both analytes to be monitored independently at different levels and without cross talk. For example, the simultaneous monitoring of lactate and glucose is of considerable interest for patient monitoring during intensive-care and surgical operations. Wilkins's group described an integrated needle-type biosensor for intravascular glucose and lactate monitoring.[101] In order to miniaturize the whole sensor and incorporate it into a hypodermic needle, the working electrode of the glucose sensor was made by electrodeposition of platinum on the needle surface, while the lactate sensor was made from platinum wire which was fixed in the needle hollow body. Palmisano et al. reported on a dual (side-by-side) Pt electrode amperometric biosensor for the simultaneous monitoring of glucose and lactate.[102] The surface coating (based on electropolymerized overoxidized polypyrrole film) resulted in excellent selectivity and no cross talk.

Wang and Zhang developed a needle-type sensor for the simultaneous continuous monitoring of glucose and insulin.[103] The integrated microsensor consisted of dual electrocatalytic (RuOx) and biocatalytic (GOx) modified carbon electrodes inserted into a needle (Figure 10) and responded independently to nanomolar and millimolar concentrations of insulin and glucose, respectively.

9. Conclusions: Future Prospects and Challenges

The enormous activity in the field of glucose biosensors is a reflection of the major clinical importance of the topic. Such huge demands for effective management of diabetes have made the disease a model in developing novel approaches for biosensors. Accordingly, for nearly 50 years we have witnessed tremendous progress in the development of electrochemical glucose biosensors. Elegant research on new sensing concepts, coupled with numerous technological innovations, has thus opened the door to widespread applications of electrochemical glucose biosensors. Such devices account for nearly 85% of the world market of biosensors. Major fundamental and technological advances have been made for enhancing the capabilities and improving the reliability of glucose measuring devices. Such intensive activity has been attributed to the tremendous economic prospects and fascinating research opportunities associated with glucose monitoring. The success of glucose blood meters has stimulated considerable interest in in-vitro and in-vivo devices for monitoring other physiologically important compounds. Similarly, new materials (membranes, mediators, electrocatalysts, etc.) and concepts, developed originally for enhancing glucose biosensors, now benefit a wide range of sensing applications.

Despite the impressive progress in the development of glucose biosensors, the promise of tight diabetes management has not been fulfilled, and there are still many challenges and obstacles related to the achievement of a highly stable and reliable continuous glycemic monitoring. Such monitoring of moment-to-moment changes in blood glucose concentrations is expected to lead to a substantial improvement in the management of diabetes. The motivation of providing such tight diabetes control thus remains the primary focus of many researchers. Clearly, success in this direction demands a detailed understanding of the underlying biochemistry, physiology, surface chemistry, electrochemistry, and material chemistry. Yet, the ultimate implementation of the new devices may rely on commercial and legal considerations rather than scientific ones.

As this field enters its fifth decade of intense research, we expect significant efforts that couple the fundamental sciences with technological advances. This stretching of the ingenuity of researchers will result in advances including the use of nanomaterials for improved electrical contact between the redox center of GOx and electrode supports, enhanced "genetically engineered" GOx, new "painless" in-vitro testing, artificial (biomimetic) receptors for glucose, advanced biocompatible membrane materials, the coupling of minimally invasive monitoring with compact insulin delivery system, new innovative approaches for noninvasive monitoring, and miniaturized long-term implants. In addition to minimally invasive and noninvasive glucose monitoring, efforts continue toward the development of chronically implanted devices (aimed at functioning reliably for periods of 6−12 months). These and similar developments will greatly improve the control and management of diabetes.

The concept of a feedback loop (sensing−delivery) system goes beyond diabetes monitoring. Such ability to deliver an optimal therapeutic dose in response to distinct changes in the body chemistry of each person offers a unique opportunity to deliver personalized medical care and dramatically change the treatment of other diseases through tailored administration of drugs.[77,78]

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