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REVIEW

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Natalie Wisniewski · Francis Moussy · W. M. Reichert

Characterization of implantable biosensor membrane biofouling

Received: 21 September 1999 / Revised: 24 November 1999 / Accepted: 19 December 1999

Abstract The material-tissue interaction that results from sensor implantation is one of the major obstacles in developing viable, long-term implantable biosensors. Strategies useful for the characterization and modification of sensor biocompatibility are widely scattered in the literature, and there are many peripheral studies from which useful information can be gleaned. The current paper reviews strategies suitable for addressing biofouling, one aspect of biosensor biocompatibility. Specifically, this paper addresses the effect of membrane biofouling on sensor sensitivity from the standpoint of glucose transport limitations. Part I discusses the *in vivo* and *in vitro* methods used to characterize biofouling and the effects of biofouling on sensor performance, while Part II presents techniques intended to improve biosensor biocompatibility.

Introduction

Viewed traditionally, sensor biocompatibility would merely describe the ill effect of the sensor on the surrounding tissue; however, more recent attention has been given to the effect of the body on the sensor [1–3]. This latter effect has been coined "sensocompatibility" [4]. One aspect of sensocompatibility is biofouling or the adhesion of proteins and other biological matter on the sensor surface. Adverse effects of biofouling can be seen with invasive and non-invasive biosensors [5, 6], immunoisolation devices [7], kidney dialysis [8–10], microdialyisis [11, 12], and in other non medically related applications such as filtration devices, desalination plants, food manufacturing, and pharmaceutical bioprocessing [13–16].

Natalie Wisniewski · W. M. Reichert (✉)
Department of Biomedical Engineering, Duke University,
Durham, NC 27708-0281, USA
e-mail: reichert@duke.edu

F. Moussy
Center for Biomaterials & Surgical Research,
University of Connecticut Health Center,
Farmington, CT 06030-1615, USA

Biosensors employ a range of transduction mechanisms – e.g. amperometric, potentiometric, conductometric and optical – each with their own unique advantages and disadvantages. The literature is thus full of papers on biosensors (over 600 papers identified on Medline alone since 1996), but nearly all of these devices cannot withstand the rigors of the *in vivo* environment, and most are suited for *in vitro* application. In fact, the *in vivo* performance of biosensors is so poor, that many companies have abandoned implantable devices all together and are focusing on *ex vivo* systems. A discussion of *in vivo* sensor limitations is given elsewhere [4].

The most widely studied and arguably the most successful implantable sensors to date are amperometric glucose electrodes. Because of their predominance in the literature and their clear clinical importance, amperometric glucose sensors are discussed most frequently in this review of biosensor biofouling. Figure 1 illustrates a model needle-type subcutaneous glucose sensor indicating sev-

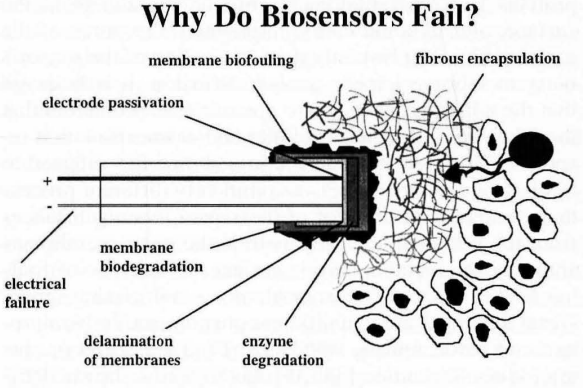

Fig. 1 Schematic illustration of a blood borne analyte exiting capillary and traversing to a needle-type glucose biosensor embedded in tissue. In addition to normal component failure – electrical failure, enzyme degradation, and membrane delamination – the sensor can fail for several physiologically related reasons, such as membrane biodegradation, electrode passivation, and reduction of analyte access due to fibrous encapsulation and membrane biofouling

eral potential sources of declining sensor signal. Glucose diffuses from the capillaries through the interstitial space and through the sensor outer membrane. This semi-permeable membrane serves as a "biocompatible" interface, which protects the underlying enzyme and electrode from immune attack. It also acts as a partial barrier to glucose, thereby allowing glucose to be the limiting reagent in its enzymatic conversion. Once glucose passes through the outer membrane, it is enzymatically converted to species that are detected by the electrode. Failure modes may be divided into two main categories: component-based failures, i.e. lead detachment, electrical short, membrane delamination; and biocompatibility-based failures, i.e. membrane biofouling, electrode passivation, fibrous encapsulation, membrane biodegradation. To date, no one has shown a convincing rank ordering of the issues leading to *in vivo* sensor failures. Some researchers advocate that biofouling of the membrane is the main problem [17–19]. Others state that enzyme degradation [20] or electrode fouling [21–23] mainly limits biosensor longevity. Still others say that biodegradation of the outer membrane is the issue [24]. The relative importance of each of these problems is further dependent on the design and construction of a particular sensor. Sensor to sensor variability is also a problem [1, 2, 25], as most sensors are produced, or at least modified, under laboratory conditions rather than tightly controlled manufacturing conditions. Some noteworthy reviews describe previous *in vivo* sensing research with emphasis on biocompatibility and sensocompatibility issues [4, 5, 26, 27]. An outstanding compilation of *in vivo* sensing technology by Fraser sheds light on many current issues facing sensor designers today [26]. In a recent article, we have reviewed the latest biosensor surface modification strategies [28]. In this review, we focus on the techniques used to measure and characterize biosensor membrane biofouling.

Membrane biofouling is a process that starts immediately upon contact of the sensor with the body when cells, proteins and other biological components adhere to the surface, and in some cases, impregnate the pores of the material [15, 29]. Not only does biofouling of the sensor's outer membrane impede analyte diffusion, it is believed that the adhering proteins are one of the main factors that modulate the longer term cellular and/or encapsulation response [30–32]. Electrode fouling, sometimes referred to as electrode passivation, is a completely different process that occurs on the interior of the sensor when substances from the body are able to penetrate the outer membranes and alter the metal electrode surface. Both types of fouling lead to the same sensor outcome – a declining sensor signal, but these are two different phenomena. *In vitro* protein and blood fouling studies [8, 17, 33–37] and *in vivo* microdialysis studies [38, 39] have clearly shown detrimental effects of membrane biofouling on analyte transport that would lead to a decreased sensor signal. Other researchers have clearly shown that electrode biofouling exists and also causes a decrease sensor signal [2, 17, 21, 40].

Fibrous tissue encapsulation is the final stage of the wound healing response to implanted, non-degradable foreign materials [41]. It generally consists of several different cellular layers surrounding the material: an inner layer of macrophages, a concentric fibrous tissue/fibroblast layer (30–100 μm), and an outer vascularized tissue layer [42]. Encapsulation has been somewhat more characterized than biofouling with respect to transport limitations. It is known that encapsulation layer thickness, vascularity, and permeability can be controlled through membrane porosity/topology. For instance, Brauker et al. showed that implanted 5 μm pore-size polytetrafluoroethylene membranes (PTFE) had 80–100 fold more vascular structures in close proximity to the membrane than did implanted 0.02 μm PTFE membranes [42]. Sharkaway et al. [43] showed that the diffusion coefficient of sodium fluorescein was 50% less through capsular tissue formed around a non-porous acetylated polyvinylalcohol implant than it was through capsular tissue around porous implants of the same material. Several other studies have confirmed that the fibrous tissue can be engineered by controlling the pore size and topology of the implant surface [44–47].

In spite of the distinction between biofouling and encapsulation, it is difficult to differentiate the deleterious effects attributed to these phenomena because both effects retard access of the sensor to analyte, and both effects are functionally intertwined. Through some *in vitro* calibration tests, however, the effects of biofouling on mass transport can be distinguished from the effects of the encapsulation tissue. Several studies have examined the permeability of membranes or sensor sensitivity before and after implantation or exposure to a biological medium [17, 19, 25, 48–52]. Upon explantation and after removal of the fibrous capsule, if the permeability of a membrane is shown to be less than before implantation, then the permeability difference can clearly be attributed to biofouling (assuming no membrane degradation occurred).

In two parts, this review intends to present the current approaches to addressing biosensor biocompatibility, particularly with regard to biofouling. Focusing on biofouling is not meant to disregard the importance of other sensor failure mechanisms; however, a thorough review of all failure modes is simply too large for the scope of this review. Part I discusses the *in vivo* and *in vitro* methods used to evaluate biofouling. Part II presents techniques intended to improve *in vivo* biocompatibility.

Part I Methods for characterizing biofouling

1 Recalibration of explanted sensors

Comparing pre-implantation and post-explantation sensor calibration results allows the mechanism of failure to be identified, at least in part. The work of Reddy et al. [17], Gilligan et al. [49] and Kerner et al. [2] provide good illustrations. In Gilligan et al., sensors that failed *in vivo* were explanted and re-tested immediately *in vitro*. Results showed that the sensors regained a significant amount of the pre-implant sensitivity. This indicates that at least a

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portion of the *in vivo* problem was due to the impedance of glucose transport through the biological medium around the sensor. High resistance of a densely fibrous, avascular capsule could have severely reduced the glucose supply to the sensor, but *in vitro*, this capsular resistance is absent. The remaining sensitivity that was not regained immediately upon explantation is most likely a result of the other issues besides encapsulation (Fig. 1). Interestingly, after 7 days of storage in saline at room temperature, the sensors regained even more of the original pre-implant sensitivity. It is possible that the explanted sensors were able to partially "unfoul" upon exposure to *in vitro* calibration conditions as biomass desorbs from the membrane. The gain in sensitivity after 7 days "strongly implicated biofouling" as a major cause of sensitivity loss [49]. If a sensor regains full sensitivity, this rules out the existence of electrical problems, membrane delamination, membrane biodegradation, or enzyme degradation for these particular sensors. Reduced signals for these sensors in the body must have been due to either membrane biofouling, electrode passivation, encapsulation, reversible enzyme denaturation, or most likely, a combination of these issues.

Kerner et al. [2] observed similar results in a small clinical study in humans. Much of the sensor signal was lost after only 6 h of implantation, but all of the signal was regained upon explantation and recalibration. To further understand the biofouling phenomenon, this group placed sensors in filtered blood. The sensors in the < 10 kDa plasma fraction showed the same fouling as observed *in vivo*. The sensor in the > 10 kDa plasma showed significantly less fouling. All fouling was reversible after 6 h. It was concluded that the main component responsible for fouling is smaller than 10 kDa [2]. This component was not identified, nor was the mechanism of fouling elucidated. It is possible that small molecules are able to penetrate into the sensor and foul the electrode, the membrane, or alter the enzymatic layer. Another study showed that filtering serum with a 3 kDa filter was required to inhibit the degradation of sensor response [53]. Fouling of electrodes with low molecular weight compounds of approximately 200 Da in size has been reported elsewhere [23, 54]. (Note: most blood plasma proteins are larger than 50 kDa).

The work of Reddy et al. [17] provides a good illustration of how pre- and post-calibration can differentiate the effects of biofouling from electrode passivation. This study used electrodes to which a 650 mV potential was applied. Various inner and outer membranes were placed over the electrodes before they were immersed in blood. These sensors were not specific for any particular analyte as no enzyme or analyte recognition element was included, rather they detected normally present reducing agents in the blood which are electrochemically active at the applied voltage. The absence of an enzyme layer, and therefore the issue of enzyme denaturation, were not issues in this study. Sensor responses were expected to be constant as long as the electrochemically active species could migrate at a constant rate to the electrode surface. Before and after blood exposure, the sensors were tested (calibrated) in a solution of catechol, which is electrochemically active at the applied potential. After the second calibration (post blood exposure), the outer membrane was separated from the inner membrane and electrode. The blood-exposed outer membrane was placed on a new, clean electrode and re-calibrated. The 'exposed' inner membrane and electrode were covered by a new unused outer membrane and re-calibrated. This model study showed that both membrane biofouling and electrode passivation occur and are detrimental to sensor output. Moreover, this study showed the relative importance of these two issues for several different membranes. By varying the composition of the inner and outer membrane, Reddy et al. [17] showed that the composition and pore size of both membranes are extremely important for reducing biofouling and passivation.

The main drawback to the pre-/post comparison is that sensors must be removed from the subject before they can be assessed. Also, *in vivo* variability and sensor-to-sensor variability is high [2, 25, 55], possibly leading to an unreliable weight attached to each failure mode. The means of *in vivo* sensor calibration further complicates the matter. Nearly all subcutaneous glucose sensor studies assume that blood glucose equals tissue glucose, and so most studies in some way compare sensor output to blood glucose concentrations. However, it is unclear whether tissue glucose levels equal whole blood glucose, plasma glucose or some fraction of these values. Researchers have reported numerous conflicting measurements of subcutaneous glucose levels. Values range from 42% of blood glucose values (validated with the three independent methods of filtration, equilibration and microdialysis) [56, 57] to 15 to 30% higher than blood glucose values (validated with the wick technique, and enzymatic glucose sensors) [58, 59]. Other researchers using the same and additional techniques have reported intermediate values between the two extremes [60–63]. Because so many researchers have obtained such varied results, perhaps there is a non-constant relationship between subcutaneous and blood glucose values. A faulty interpretation of subcutaneous glucose concentrations can lead to serious errors in *in vivo* sensor calibration.

It is well established that changes in glucose measured subcutaneously lag behind changes in blood glucose levels by as much as 15 min [64]. Further, some researchers perform glucose load studies, while others monitor normal resting conditions, reporting one or more of the following – sensitivity, lag time, current output, decreases in current output, potential, decrease in potential over time.

Standardization of sensor studies would greatly facilitate inter-study comparisons. The studies of Reddy et al. [17], Moussy et al. [55] and Gilligan et al. [49] are notable examples of thorough sensor characterization. In these studies the following items were reported: 1) a precise description of inner and outer sensor materials and detailed sensor design; 2) pre-implant and post-explant sensor output/sensitivity reported over a range of calibration fluid concentrations; 3) comparison of sensor output to mea-

sured blood values based on initial calibrations; and 4) a clear statement of the measurement range, measurement standard deviation, and number of sensors tested.

2 Microscopic techniques

Arguably the most straightforward method of assessing the existence of biofouling is by direct visualization using either light microscopy, scanning electron microscopy (SEM) or transmission electron microscopy (TEM). The use of light and electron microscopy in biomaterials evaluation has been reviewed recently [65–67]. Light microscopy, with its resolution around 1 μm [67], is used to assess the overall tissue reaction to implanted sensors or sensor membranes. Usually, light microscopy is performed on explanted, fixed tissue which has been stained (e.g. with hematoxylin and eosin), sectioned and mounting on microscope slides. Ertefai and Gough [68] and Moatti-Sirat et al. [69] provide excellent examples of using light microscopy to characterize the nature of the tissue surrounding the sensor tip. Most commonly with sensors, this technique is used to assess the degree of inflammation, the types of cells present, the level of vascularity, the integration of tissue with the sensor surface, and the thickness and fibrosity of the encapsulation tissue.

SEM and TEM are ultra high vacuum techniques of much higher resolution, usually on the order of microns going down to sub-nanometer resolution [65, 67]. Samples are usually prepared for TEM analysis by replication of the surface by molding or by thin sectioning [67]. In SEM, the sample is fixed, critically point dried, and coated with a thin layer of metal, usually gold and/or platinum. Secondary electrons emitted from the illuminated sample produce a surface relief image that reveals excellent detail of the surface of the specimen [65, 67]. Quinn et al. [70] and Moussy et al. [71] used SEM to show substantial differences in the morphology of material adhering to hydrogel and Nafion coatings after several days of implantation. Zhang et al. [72] showed sensor surfaces after contact with blood. Other studies evaluated the surface appearance of membranes intended for sensors, (though not attached to sensors) after subcutaneous implantation in rats [48, 70], and after exposure to protein solutions [38, 52], plasma [73, 74], and whole blood [51, 75].

Thin section TEM in conjunction with gold or silver colloids has been used to visualize adherent proteins on the surface, and in the pores of polyvinylidene fluoride, polysulfone, and reconstituted cellulose filter membranes [15, 76]. In one study, the effect of phosphorylcholine-based treatments on albumin fouling were visualized using protogold particles chemically linked to adherent albumin molecules [15]. In another study, silver or gold colloids were simply filtered though fouled membranes and then visualized without chemical coupling [76]. Greater biofouling reduced the permeability of the membrane, causing more gold or silver to remain in the membrane. For unfouled membranes, the particles passed through and were not able to be visualized as in the fouled membranes. The resulting images allowed a clear visualization of the pore structure of the fouled material. This technique is applicable to porous membranes, but would not be appropriate for non-porous membranes.

3 Characterization of implanted membrane discs

Valuable information on sensor membrane permeability has been obtained by separating the membrane from the sensor, implanting the membrane, and then analyzing the explanted membrane *in vitro*. Again, the major drawback here is that the specimen must be explanted to be characterized. Moussy and Mercado implanted discs of Nafion sensor membranes into rats [29, 48]. Membrane permeability was assessed in a diffusion chamber before implantation and after explantation, which occurred in one-week intervals up to 4 weeks. It was found that the permeability of Nafion went up after one week but then declined in the three successive weeks. In this study, it was also shown that biofouling can occur not only on the surface, but also within the membrane itself. Calcium phosphate deposits were detected with a florescent dye for Ca^{2+} in perfluorosulfonated ionomer Nafion membranes after 4 weeks implantation in a rat. The mineralization of these commonly used Nafion membranes causes cracking and decreases glucose permeability starting at one week after implantation [29, 48]. By isolating the membrane from the rest of the sensor, this technique provides a good method of comparing the permeability loss of different membranes without the confounding effects of other sensor issues. A drawback of this method is that the membrane-tissue interface can be assessed only if the tissue is disrupted, thus allowing no further characterization, and limiting membrane assessments to only one time point.

A similar approach was taken by Sharkaway et al. [43] to determine the effect of membrane topology on the analyte diffusion and perfusion in the tissue that encapsulates implanted acetylated polyvinylalcohol and expanded polytetrafluoroethylene specimens. Rather than characterizing the implanted membrane material, this study characterized analyte transport through the surrounding encapsulation tissue. It was found that the less fibrous and more vascularized encapsulation tissue that surrounds porous specimens was more permeable to a glucose-mimicking substance than was the thin but highly fibrous and avascular tissue that forms around smooth specimens.

An interesting hybrid approach was employed by Lindner et al. who examined the inflammation reaction to K^+ and pH ion selective membrane sensors using the stainless steel cage implant model of Marchant and Anderson [77]. The effect of sensing membrane plasticizer content and the application of a polyhydroxyethylmethacrylate (PHEMA) hydrogel coating were examined by placing specimens in cages and implanting them in the dorsal rat subcutis. Using empty cages as controls, this model exposes the implant to exudate and inflammatory cells, but protects the device from direct contact with wound healing tissue. In addition to examining cellular

and protein accumulation on the explanted sensor, this system allows one to periodically withdraw aliquots of exudate from within the cage, and thus quantitatively and temporally assess the inflammatory reaction to the implant. It was found that membranes plasticized with certain surfactants evoked a more pronounced inflammatory response than non-plasticized membranes [77].

4 Microdialysis

Microdialysis offers an excellent means of characterizing analyte transport across membranes *in vivo* in real time. In general, microdialysis consists of a single implanted semipermeable hollow fiber whose lumen is perfused with physiological fluid (perfusate) [78]. Molecules in the fiber lumen and in the surrounding tissue space undergo diffusional exchange across the membrane in a manner very much like a small diameter blood vessel. Owing to the molecular weight cut off of the membrane, only molecules up to a certain size (just as with biosensor membranes) will diffuse from the tissue interstitial fluid into the flowing perfusate. The "exchanged" fluid exiting the implanted fiber (dialysate) is collected and analyzed for molecules reflecting the composition of the surrounding tissue. Analytical techniques such as flourimetry, HPLC, or electrochemical sensing may be applied to analyze the sampled dialysate. Just as with a biosensor, if proteins and other substances foul the microdialysis membrane, less analyte will diffuse through the microdialysis membrane. Microdialysis membranes can be made from or coated with the same materials as biosensors membranes, so this technique allows the issues of the membrane-tissue interface to be isolated from other sensor issues (enzyme degradation, electrical failures, etc.) that typically confound the study of biosensor membranes or coatings.

Microdialysis has been used mainly to elucidate the chemistry/physiology of specific tissues over short periods of time. The reduction of analyte recovery over time through microdialysis probes has been noted, but has been viewed as a limitation of the technique. Though not the topic of their study, researchers have reported decreased analyte transport into and out of various microdialysis membranes [79–81]. One pharmacokinetics study which used intravenously implanted probes in rats showed about 20% less delivery of a drug analog on day 10 compared to day 2 after implantation of a acrylonitrile/sodium methyl sulfonate copolymer membrane [79]. Another drug delivery study in rabbit vitreous humor reported the glucose transport through implanted polyamide membranes after 6–10 days of implantation to be approximately 40% less than in the first 5 days after implantation [80]. A third study to assess caffeine levels in various tissues showed large differences in transport losses between 24 and 72 h implantation in different tissue types [81]. The type of dialysis membrane was not specified, however a 47% decrease of transport in brain, a 40% decrease of transport in muscle and an insignificant reduction of transport across the implanted membrane in blood over the implantation time was reported. Other studies have also reported substantial losses in analyte transport over time through microdialysis probes in various tissues over various lengths of time [12, 82, 83]. The previous studies were not designed to study biofouling, however they reveal some interesting effects of membrane tissue interactions on transport through implanted membranes.

A few sensor researchers have used microdialysis to better understand how transport through different membranes is affected by the interface of the membrane with a biological medium [38, 84, 85]. Hashiguchi et al. [85] found that 7 day exposure to 10, 30 and 50 mg/mL albumin caused approximately an 11, 17, and 20% loss of glucose transport through a 50 kDa cut off regenerated cellulose membrane. *In vivo*, the same membrane had a gradual decrease over 7 days with *in vivo* calibration factors decreasing to 65.7% of the value on the first day. Ishihara et al. found that methacryloxyethyl phosphorylcholine (MPC) coating on cellulose hollow fibers allowed for much greater stability of glucose permeability over 14 days of submersion in bovine serum albumin solution [38]. Unmodified cellulose becomes nearly impermeable to glucose by day 14; whereas MPC modified cellulose has only a 20–25% decrease in recovery. Based on modeling of experimental *in vivo* microdialysis results, another study found that the tissue reaction to and/or the biofouling of several membrane materials caused significant increases in resistance to glucose flux over 8 days of implantation [86]. More studies of this type can lead to a better understanding of transport issues in sensor membranes. It is likely that quantitative microdialysis studies of membrane/tissue interactions will increase as the technique gains greater acceptance, and as the transport modeling improves [11, 87].

5 Gravimetric methods

As biofouling progresses, the mass of a given membrane will increase. One may quantify the amount of biofouling by comparing the total membrane mass before and after biological exposure. Kidney dialysis membranes (many of which are the same materials used in biosensor membranes) have been characterized according to weight gain after exposure to blood, plasma, or individual protein solutions. For instance, surface coverage for polycarbonate and polyacrylonitrile membranes was around 1 $\mu g/cm^2$ and 75 $\mu g/cm^2$, respectively [34]. A similar study was performed with plasma rather than blood, showing that polysulfone specimens absorbed far more protein mass than did polymethylmethacrylate or polycarbonate specimens [32]. Although biosensing and kidney dialysis are two different fields, both involve transport of an analyte across a membrane that is in contact with a biological medium. Both are negatively affected by biofouling, and since many membrane materials are common to both fields, the extensive literature of the dialysis field may shed some light on biofouling in the sensor field. For this reason, several references are made here and in the fol-

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lowing sections about biofouling of kidney dialysis membranes.

Quartz crystal microbalances (QCM) are oscillating piezoelectric crystals with a resonant frequency that shifts in proportion to the mass that accumulates on the crystal face. QCM allow continuous observation of mass gains while the biofilm accumulates *in vitro*. Using a gold coated QCM modified by self assembled monolayers, it was found that surfaces of ethylene glycol oligomers were more resistant to BSA absorption than acrylamide oligomers, and acrylamide was more resistant than N, N-dimethylacrylamide surfaces [88]. The benefit of the QMC method it that it is very sensitive to small changes in mass. A drawback is that the sensor surface of interest must be able to be immobilized on a quartz crystal, which is not always possible. Also, the technique is restricted to *in vitro* usage.

6 ATR IR spectroscopy and ellipsometry

Attenuated total reflection infrared spectroscopy (ATR IR) and ellipsometry fall under the general rubric of surface analytical techniques [89]. These techniques as applied to biomaterials research have been reviewed recently [90, 91]. The vast majority of their application has involved studying protein adsorption, and they are applicable to the study of the biofouling of some biosensor membranes that can be appropriately applied to the test system. ATR IR measures the absorption of a totally reflected infrared beam at the solid/liquid interface. By coating an IR transparent substrate with the material of interest, one can monitor in real time the accumulation of proteins and cells by monitoring the increase in IR absorption. A major advantage of ATR IR is the ability to follow specific adsorption bands, which can be interpreted as changes in the structure and composition of the adsorbed proteins. Recently, this technique has been used to characterize bacterial biofilm growth on surfaces [92], and some of the first quantitative studies of blood/materials interactions were made using this technique [93]. It is limited to *in vitro* studies, and it is applicable only to membranes that can be applied in a thin enough layer to permit penetration of a given wavelength for the detection of adhering proteins.

Ellipsometry uses changes in the phase and amplitude of a reflected beam to calculate changes in the thickness and refractive index of a film deposited on a reflective surface, usually a metal. Several studies have used ellipsometry to study protein and other interactions on surfaces [91]. From the standpoint of membrane design for biosensors, ATR-IR and ellipsometry are good tools for initial assessment of a potential sensor coating material, but they are not directly amenable to assessing the performance of intact membranes already on a sensor.

7 Protein identification methods

Protein elution is a long-standing and reliable method for identifying the proteins that bind to solid specimens [94]. Kuwahara and Markert [32] eluted proteins from biofouled kidney dialysis membranes by SDS buffer, and separated the eluted proteins into bands by gel electrophoresis. A few membrane specific protein bands were present, giving a possible reason for potential difference in the biological activity of these membranes *in vivo*. It was shown that all membranes adsorbed albumin, but polysulfone and polyacrylonitrile did so to a lesser degree. Polysulfone and polyacrylonitrile, however, adsorbed the most protein overall. It is interesting to note that in the field of kidney dialysis, polysulfone and polyacrylonitrile are noted to be more biocompatible than the other membranes [32]. In a similar study, protein was eluted from Cuprophan and polymethylmethacrylate (PMMA) hemodialysis fibers, analyzed by electrophoresis and immunoblotted. PMMA, but not Cuprophan, showed the presence of glycoproteins derived from platelets. Cuprophan eluents were richer in albumin, IgG, and antithrombin III than were PMMA eluents [30]. A study of this kind has not been conducted for implanted biosensors; however, for future design of biofouling repellant biosensor membranes, it would be valuable to understand which proteins are actually fouling various membranes. One consideration is that lipids and other biological molecules besides proteins may be fouling implanted membranes [95], and so identification techniques other than electrophoresis would be needed.

Histochemical techniques employ specific staining reactions to identify particular aspects of histological specimens, such as a particular cell type or protein [96]. Immunohistochemistry uses the specificity of "tagged" monoclonal antibodies to perform this identification. Immunohistochemical techniques have been used to determine albumin, fibrinogen, fibronectin, IgG and collagen around polytetrafluoroethylene and other implants [97, 98]. These techniques nicely characterize the distribution of the various proteins in the capsular layer around implants using light microscopy. However because the biofouling layer is thin compared to the capsule, the interfacial protein composition is difficult to analyze by conventional histological methods. Estimates of thickness of protein fouling layers on various materials are in the range of 0.5 μm [34] to 20 nm [99]. Red blood cells are approximately 8–9 μm in diameter and a giant macrophage cell may be as large as 20 μm. The capsule in comparison is much larger, starting at the surface and radiating outward 100 to 200 μm.

8 Membrane permeability studies

Several thorough studies have been conducted to characterize the reduced transport through the biofouling layer on membranes with previous blood contact. Most of these studies have focused on convective flow from the standpoint of kidney dialysis and ultrafiltration. Hydraulic permeability, sieving coefficients, solute flux, and diffusive permeabilities have all been characterized for various fouled and non-fouled dialysis membranes [8, 33–37].

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Sakai provides a thorough review of all these characterization methods and also discusses a variety of models to describe membrane transport [100]. The extensive literature on membrane-protein interactions in the kidney dialysis field may contribute to our understanding of biosensor membranes, however, the relationship between biofouling in dialysis and sensors has not been firmly established.

Part II Strategies to improve sensor biocompatibility

Bioanalytical sensors have over the years proved to be inadequate for long term *in vivo* applications, with membrane biofouling playing a significant role in sensor instability. A companion paper reviews sensor modifications intended to reduce *in vivo* sensor membrane biofouling including hydrogels, phospholipid-based biomimicry, flow-based systems, Nafion, surfactants, naturally derived materials, covalent attachments, diamond-like carbons and topology [28]. However, biofouling is just part of the *in vivo* performance problem, and such modifications should be used in combination with approaches for addressing other aspects of sensor biocompatibility. The following section presents sensor modifications intended to reduce protein adsorption, increase integration of the sensor with the surrounding tissue, and encourage tissue responses such as angiogenesis.

1 Modifications to reduce protein adsorption

One simple strategy to improve sensor biocompatibility would be to reduce protein adsorption. This can be achieved by modifying the surface of the sensor or adding a new layer of a special material [28]. Simple surface modifications can be performed by creating functionalities such as hydroxyl, carboxyl, amine, sulfonate or phosphate groups on the surface [101]. As a result, adsorption of some molecules may be reduced. However, because so many different proteins are present at the sensor/tissue interphase (i.e. the transitional region between the sensor and its surrounding tissue), it is unlikely that all total resistance to protein adsorption could be achieved. It is also not known which proteins lead to biocompatibility or bioincompatibility [31]. Therefore, it is unlikely that a single, simple surface modification alone will suffice.

One approach to surface modification of a biosensor attempted by Quinn et al. [70] consists of incorporating poly(ethylene glycol) (PEG) into a polyhydroxyethylmethacrylate (PHEMA) membrane. The PEG chains (also often called polyethylene oxide (PEO)) tend to line up parallel to each other and perpendicular to the surface to present a water rich phase that resists penetration by many proteins [102]. Quinn et al. demonstrated that PEG in the outer membrane induced less fibrous encapsulation after subcutaneous implantation in rats, compared to the same membranes without PEG, but did not report the resulting change in sensor performance [70, 103].

Another strategy to reduce protein adsorption has been the coating of the surface of biosensors with phosphorylcholine (PC) groups to mimic the red blood cell surfaces and therefore to transfer the non-thrombogenicity of the red blood cells to the biosensor's surface. The diminution of protein adsorption on PC coated surfaces has been demonstrated [38, 73, 104–106]. The antifouling characteristics are believed to be due to the ability of the PC groups to render the surface extremely hydrophilic, so that proteins have difficulty adsorbing on the surface because of the layer of bound water. Using this approach, Nishida [18] showed that subcutaneously implanted biosensors could measure the glucose concentration in humans for up to 14 days. However, *in vivo* re-calibrations were required after 7 days due to the loss of sensitivity, thus limiting the benefit of this approach for long-term implantation. Other methods to reduce protein adsorption include the use of a very slow flow of phosphate buffer over the tip of the sensor [107], so-called "inert" materials such as diamond-like carbon that have reduced material-tissue interactions [51], and modification of membrane with surfactants [17].

Since the loss of sensor function caused by the tissue response to the implant is a complex phenomenon, it is likely that strategies more sophisticated than simply reducing protein adsorption are needed. For many implantable biosensors, the loss of function is also a consequence of inflammation and fibrosis with loss of vasculature resulting from the tissue trauma and long term foreign body response caused by the sensor implantation and by reactions within the tissue. The next generation of implantable biosensors may find it useful to borrow new strategies from the rapidly expanding field of tissue and

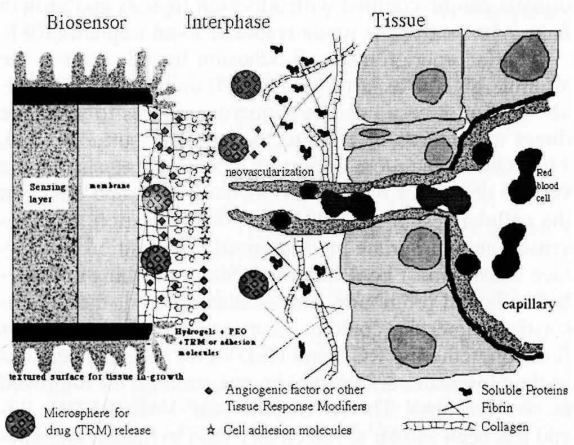

Fig. 2 Schematic illustration of potential options for modifications of an implantable biosensor to improve its biocompatibility. Depicted are proposed interactive surface hydrogels with bound adhesion ligands and TRM, biodegradable TRM-release systems, and surface texturing. Incorporating some or all of these new systems should enhance the sensor's function and lifetime *in vivo* by suppressing inflammation and fibrosis as well as enhancing blood vessel density around the sensor

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molecular engineering to suppress inflammation and fibrosis, as well as enhance neovascularization around the implanted biosensor. To achieve this goal, three complementary strategies could be used: surface modification/functionalization of the biosensor utilizing hydrogels with adhesion ligands and bound tissue response modifiers (TRM); local drug delivery systems containing TRM to provide long term delivery of these factors within the sensor/tissue interphase; and physical modifications to influence the tissue response to the implanted biosensor. These strategies discussed below are summarized in Fig. 2.

2 Hydrogel modifications employing adhesion ligands and growth factors

Modifying the surface of an implantable biosensor with adhesion ligands and growth factors may provide a better integration of the sensor within the tissue. However, surface modifications of implantable chemical biosensors are more challenging than what is required for non-interactive implants (e.g. catheters) since sensors must maintain appropriate analyte transport through the multi-layer coatings on the sensor element. There is a minimum analyte diffusion rate above which the sensor will respond adequately to fluctuations in the analyte concentration. Any additional coatings applied to the sensor must therefore be either ultra-thin or be sufficiently porous to have minimal impact on the analyte diffusion flux. For this reason, highly water-swollen surface hydrogels that have minimal impact on the diffusion rates of small molecules could be used to provide a support for surface modifications of implantable biosensors. Furthermore, hydrogels are already widely used in a variety of biomaterial applications, most notably in contact lenses, and it has been shown that hydrogels can be coupled with adhesion ligands and growth factors to control the tissue response to an implant [108].

The incorporation of cell adhesion ligands such as the arginine-glycine-aspartic acid (RGD) motif into biomaterials is being investigated by numerous groups to stimulate direct attachment of cells to the material surfaces [109, 110]. One of the more important recent findings is that the surface density of binding-motifs has a profound effect on the cellular response [111–114]. For application to a biosensor, an appropriate binding-motif composition and surface density must be determined. Since attaching endothelial cells and promoting neovascularization at the biosensor-tissue interface could in theory improve the biosensor's function, tethering RGD and REDV cell adhesion peptides to the biosensor surface may be one strategy for improved *in vivo* response. The ARG-GLU-ASP-VAL (REDV) ligand has been shown to selectively bind to human endothelial cells, but not to bind to smooth muscle cells, fibroblasts or blood platelets when used in an appropriate amount [115]. A specific adhesion ligand, such as REDV, could thus be used to coat the biosensor with one desired cell type while preventing other cells from adhering.

Other synthetic and naturally occurring mediators that control tissue and cell responses, such as inflammation, fibrosis and neovascularization could be used. These mediators, termed collectively herein tissue response modifiers (TRM), include cytokines, anti-inflammatory drugs, growth factors (including heparin), neutralizing antibodies, hormones and metabolic intermediates [116]. The control of inflammation and repair (fibrosis) in tissue has classically focused on the use of steroidal and non-steroidal anti-inflammatory drugs [117–120]. However, long-term systemic use of these drugs is not desirable since major side effects can develop with time. Local immobilization of such drugs may be advantageous to their long-term application. One of the most rapidly developing areas of research has been in the area of growth factors. Specific growth factors have been effective in controlling fibrosis (e.g. transforming growth factor beta, TGFB), and promoting new blood vessel formation in both wound healing and cancerous tissues (e.g. VEGF). Thus, incorporation of growth factors into hydrogels on the surface of glucose sensors would likely provide a means to control the sensor-tissue interface. Of particular interest for a biosensor is VEGF, vascular endothelial growth factor, which selectively promotes proliferation of endothelial cells and not fibroblasts. VEGF could be chemically tethered to the hydrogels using a method similar to the one used by Griffith's group to tether the epidermal growth factor (EGF) [121, 122]. EGF was found to retain an active conformation when tethered to a solid substrate, suggesting that properly tethered VEGF may also retain activity to promote the neovascularization desired for biosensor applications. An intermediate protein-resistant layer such as PEO could be incorporated in the hydrogel or placed between the hydrogel and the attached adhesion ligands/TRM in order to reduce the non-specific adsorption of proteins that could mask the adhesion ligands/TRM. Incorporation of intermediate lengths of flexible PEO sequences may provide sufficient mobility to allow proper access to the attached molecules [121] yet prohibit fouling molecules from gaining access to the underlying surface.

3 Local drug delivery strategies

Site specific, controlled release delivery of TRM could be used alone or in combination with the above surface modifications to help control the tissue response to an implanted biosensor. Again, the objective would be to suppress inflammation and fibrosis as well as to enhance neovascularization around the sensor. This mode of local delivery has the advantages of reducing or eliminating systemic side effects and improving the therapeutic response through appropriate controlled dosing at the site. Various TRM (such as dexamethazone, ketoprofen, transforming growth factor alpha (TGFA), anti-fibroblast antibody, VEGF, etc.) could be delivered depending on the duration and site of implantation. Numerous strategies to deliver TRM around the sensor could be used. For example, the sensor could be designed with a small reservoir from which the TRM would be released. This approach is being used to deliver dexamethasone (an anti-inflammatory sub-

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stance) at the tip of pacemaker leads [123]. The sensor could also incorporate a layer that would slowly degrade *in vivo* and thus release the drug. Perhaps, the easiest strategy could be to use biodegradable microspheres. Microspheres are micron sized spherical particles, typically prepared using natural or synthetic polymers such as polylactic-glycolic acid (PLGA). Microspheres can have drugs incorporated in the core or dispersed throughout the polymer matrix [124]. Microspheres have been utilized for site specific, controlled delivery of both small molecular weight drugs (e.g. dexamethasone [125, 126]) and proteins [127–129] including growth factors [130]). Sustained release preparations of vascular growth factors have been reported using similar approaches [131–134]. Such microspheres could be placed adjacent to the sensor or incorporated into the surface hydrogel on the sensor (Fig. 2) to deliver TRM and thus control tissue responses to injury at the site of the biosensor implantation. This proposed strategy has yet to be incorporated in a biosensor.

4 Physical modification strategies

The surface of the implanted biosensor could also be physically modified to improve its biocompatibility. The surface topography (roughness, texture and porosity) of a material has been shown to influence the tissue response to an implant. Von Recum's group described an optimal tissue response for a material with 1–2 μm pore size [135]. Smaller pore sizes caused poor adhesion and increased inflammatory response. Larger pore sizes allowed in-growth and anchorage but caused a more severe foreign body reaction. Brauker et al. demonstrated that the pore size had a significant effect on the vascularization of PTFE implants [42]. These results were confirmed by Sharkaway et al. who also calculated the barrier to diffusion of the fibrous tissue encapsulating porous and nonporous materials [43–45]. One could thus use these findings to modify the topography of a biosensor to obtain a more desirable tissue response. Although this has yet to be demonstrated, an implantable biosensor with a textured surface that would allow tissue in-growth for anchorage as well as the development of a capillary network should be able to more accurately detect changes in the analyte concentration.

Conclusions

A recent quote from George Wilson and Gerard Reach [136], two experts in the field of glucose sensors, points out the persistent ambiguity of the biosensor biocompatibility problem. "It is likely that an additional layer is formed around and/or inside the outer membrane (of the implanted sensor) limiting glucose access to the electrode and decreasing sensitivity. This additional layer is apparently loose and disappears progressively during rinsing, thus accounting for the partial reversibility of the decrease in sensor sensitivity observed after implantation. In this respect, the presence of the additional layer covering the whole surface of explanted sensors, which disappears after sensor rinsing, may be relevant, although it is hazardous to speculate that this layer (the chemical nature of which remains to be determined) represents the physical barrier to glucose". In offering a solution they simply say "It is likely that this problem could be resolved by modifying the outer sensor membrane, which would alter these interactions, the nature of which remains to be determined". In this review, we noted the various approaches used to characterize biosensor biofouling, as well as offering some promising strategies for alleviating the deleterious effects of sensor implantation. However, as pointed out by Wilson and Reach, the phenomenon of biosensor biocompatibility has not yet been adequately understood, and despite recent advancements, a reliable solution remains elusive.

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