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Bioactive Electroconductive Hydrogels: The Effects of Electropolymerization Charge Density on the Storage Stability of an Enzyme-Based Biosensor

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Abstract Electrode-supported hydrogels were conferred with the biospecificity of enzymes during the process of electropolymerization to give rise to a class of bioactive, stimuli-responsive co-joined interpenetrating networks of inherently conductive polymers and highly hydrated hydrogels. Glucose responsive biotransducers were prepared by potentiostatic electropolymerization [750 mV vs. Ag/AgCl (3 M KCl)] of pyrrole at Poly(hydoxyethyl methacrylate)-based hydrogel-coated Pt micro-electrodes (Φ=100 μm) from aqueous solutions of pyrrole and glucose oxidase (GOx; 0.4 M pyrrole, 1.0 mg/ml GOx) to 1.0 and 10.0 mC/cm^2. Polypyrrole was them over-oxidized by cyclic voltammetry (0–1.2 V vs. Ag/AgCl, 40 cycles in PBKCl, pH=7.0). Biotransducers were stored at 4 °C in PBKCl for up to 18 days. Amperometric dose–response at 0.4 V vs. Ag/AgCl followed by Lineweaver–Burk analysis produced enzyme kinetic parameters as a function of electropolymerization charge density and storage time. Apparent Michaelis constant (K_{Mapp}) increased from 18.6–152.0 mM (1.0 mC/cm^2) and from 2.7–6.1 mM (10.0 mC/cm^2). Biotransducer sensitivity increased to 21.2 nA/mM after 18 days and to 12.8 pA/mM after 10 days for the 1.0 and 10.0 mC/cm^2 membranes, respectively. Maximum current, I_{max}, also increased over time to 2.7 nA (1.0 mC/cm^2) and to 170 pA (10.0 mC/cm^2). Electropolymerization of polypyrrole is shown to be an effective means for imparting bioactivity to a hydrogel-coated microelectrode. GOx was shown to be stabilized and to increase activity over time within the electroconductive hydrogel.

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Introduction

Hydrogels are 3-D polymer networks of highly hydrophillic monomer that are commonly employed for imparting biocompatibility to implantable devices. Polymeric hydrogels are being molecularly engineered to serve plural functional roles; (a) as bioreceptor hosting membranes of enzyme-based implantable biosensors [1], (b) as biomimetically inspired biocompatible coatings on stents and other implantable bionic devices [2], and (c) as stimuli-responsive membranes [3] that directly transmute chemical potential energy into electrical signals via incorporation of one-dimensional conductive electroactive polymers such as polypyrrole (PPy) [4] and polyaniline (PAn) [5]. Electroconductive hydrogels (ECH) of Poly (hydoxyethyl methacrylate) poly(HEMA)-PPy have shown in vitro biocompatibility through growth and proliferation of PC12 neuronal cells and RMS 13 muscle fibroblasts [6, 7]. ECH have also been shown to provide effective screening of endogenous interferents [8] when used as the biorecognition membranes of amperometric enzyme biotransducers [9]. Poly(HEMA)-PPy ECH have been used as the enzyme hosting membrane in implantable enzyme-amperometric biotransducers [10].

Electropolymerization produces controllable thin films of inherently conductive electroactive polymers (CEP) deposited at electrode sites [11, 12]. During formation such polymers entrap counter anions that serve as “dopants”. In the presence of enzymes, which are often negatively charged, electropolymerization leads to formation of an enzyme-enriched membrane layer deposited directly on the electrode [13]. Electropolymerization to entrap enzymes at metallic electrodes, first explored by Foulds and Lowe [14] and Yamamoto et al. [13], had limited success. Two major challenges exist with this approach. Firstly, the rapid denaturation of the enzyme in the dense hostile environment of the CEP; secondly, the high background currents associated with the CEP at the electrode. Two major innovations have been introduced to address these limitations. Firstly, the electropolymerization is performed at hydrogel-coated electrodes thereby placing the enzyme within a water-rich milieu that sustains its bioactivity [15]. Secondly, following electropolymerization, the electroactive polymer is over-oxidized [16], eliminating its contribution to a high background amperometric current during interrogation. These innovations offer the advantage of high efficiency of enzyme loading, ease of preparation, stability of the enzyme/CEP/hydrogel membrane, interference suppression [8], and enables control of the hybrid polymer membrane properties. Electropolymerization into hydrogel-modified electrodes has since been shown to be a convenient method to immobilize multiple enzymes, sequentially to produce layered enzyme structures and also at different electrode surfaces of implantable multi-analyte biotransducers. Glucose oxidase (GOx) and lactate oxidase were separately immobilized via electropolymerization of pyrrole into biocompatible hydrogels to produce bioactive electroconductive hydrogels suitable for use as amperometric biotransducers with clinically relevant properties.

Poly(HEMA)-based hydrogels have recently been developed for intramuscularly implantable biosensors [2, 4, 17–21]. These materials are designed to be biomimetic through the incorporation of 2-methacryloyloxyethyl phosphorylcholine (MPC), a monomer containing the phosphorylcholine pendant group typically found as the zwitterionic head group of the outer leaflet of the plasma membrane [2]. PPy has been shown to be an effective means to immobilize enzymes through electropolymerization, modify sensitivity to analytes by lowering the electrical impedance from the solution phase to the electrode, and screen for negatively charged, electrochemically reactive interferents [8, 17].

Over time, significant changes to biosensor performance in terms of sensitivity, dynamic range, response time and limits of detection can be expected. Biosensors can be optimized as a function of incorporated hydrogel and polypyrrole content to maximize these performance criteria. The long-term effects of polypyrrole incorporation on biosensor performance are explored. In addition to the materials selection, longevity of biosensor systems is also a function of their manufacturing, packaging, and storage. During storage, a stable environment for maintaining enzyme activity and hydrogel conditioning requires optimization of buffer type, pH, ionic strength, and temperature. This paper outlines the effects of storage on the apparent enzyme kinetic parameters of a biosensor system utilizing hydrogels designed for intramuscular implantation.

Experimental

Chemicals and Reagents

Pyrrole monomer (reagent grade 98%+), GOx (E.C. 1.1.3.4 from *Aspergillus niger*), 2-hydroxyethyl methacrylate (HEMA), tetra(ethylene glycol) diacrylate (TEGDA, technical grade), poly(ethylene glycol) (400)monomethacrylate (PEGMA), *N*-[Tris(hydroxymethyl) methyl]acrylamide (HMMA, 93%), polyvinylpyrrolidone (pNVP), 2-(dimethylamino) ethyl methacrylate (DMAEMA, 98%), the photo-initiator 2,2-dimethoxy-2-phenylacetophenone (DMPA, 99%+), β-D(+)-glucose, ascorbic acid, sodium dihydrogen phosphate, disodium hydrogen phosphate, potassium chloride, sodium hydroxide, and sulfuric acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). 2-(Methacryloyloxy)ethyl 2-(trimethylammonio) ethyl phosphate (MPC) was purchased from Tokyo Chemical Industry Co., Ltd. The HEMA, methacrylate, and diacrylate reagents were passed through an inhibitor removal column (Sigma-Aldrich) for removal of the polymerization inhibitors hydroquinone and monomethyl ether hydroquinone before using them in the preparation of the hydrogel cocktail. Pyrrole monomer was purified by double passage through an alumina silicate column. Solutions were prepared in deionized water prepared by purifying distilled water through a Milli-Q plus (Millipore Inc.) ultrapure water system. The glucose stock solution of 1.0 M was prepared and allowed to mutarotate overnight.

Electrochemical Measurements

Electrochemical experiments were performed using a BAS-100B/W (West Lafayette, USA) Electrochemical Analyzer with a BAS PA-1 preamplifier module used to amplify the current and to filter out noise. All experiments were carried out in a three-electrode configuration with the platinum microelectrode (West Lafayette, USA) as the working electrode (W.E.), a Ag/AgCl 3 M KCl reference electrode (ABTECH Scientific, Inc., Richmond, VA) and a platinum mesh counter electrode.

Preparation of Hydrogels and Electrode Cleaning Procedures

A 3 mol% TEGDA cross-linked standard hydrogel cocktail was prepared according to a previously described protocol [22] by mixing HEMA, TEGDA, PEG(400)MA, MPC, HMMA, p(NVP), DMAEMA, and DMPA in typical ratio 78:3:5:1:5:2:5:1 mol%. A 1:1 (*v*/*v*) solution of ethylene glycol/water corresponding to 20% of the volume of the monomer cocktail was then added to the monomer cocktail and stirred overnight under UV-free

conditions. Initially, the working platinum electrode (BAS, Φ=100 μm) was mechanically polished with 0.05 μm alumina for 10 min and then washed with nanopore water and acetone, respectively, in order to expose a fresh platinum surface. Hydroxyl groups were introduced to the glass surrounding the platinum surface by RCA clean treatment (DI-water/hydrogen peroxide/ammonium hydroxide, 5:1:1 ratio) for 5 s followed by immediate and thorough rinsing with DI-water. The electrode was then immersed in 0.5 M sulfuric acid, made the working electrode of a three-electrode electrochemical cell and was electrochemically cleaned by sweeping the potential between −0.20 and 1.40 V (vs. Ag/AgCl, 3 M KCl) at 100 mV/s until a steady-state cyclic voltammogram was obtained. This electrochemical pretreatment followed by rinsing with nanopore water several times to ensure good repeatability of the electrode surface.

Surface Modification and Application of Hydrogel to Platinum Microelectrode Surface

Following cleaning the bare platinum microelectrode surface was silanized by incubation in a 0.1 wt.% solution of 3-aminopropyl-trimethoxysilane in anhydrous toluene for 1 h followed by thorough rinsing with toluene and water. The electrode surface was subsequently functionalized with acrylate groups by incubation in a solution of 1 mM acryloyl(polyethyleneglycol)-*N*-hydroxysuccinamide (ACRL-PEG-NHS, MW 400) in 0.1 M HEPES buffer, pH=8.5 for 2 h. Functionalization of electrodes was followed by ultrasonication in DI-water for 5 min and drying with ultrahigh purity nitrogen. The microelectrode was dipped in the 3 mol% TEGDA cross-linked p(HEMA)-based hydrogel cocktail which had been sonicated and sparged with nitrogen. The thin film was then cross-linked with UV light for 5 min using a UV cross-linker (CX-2000 Crosslinker, UVP, Upland, CA, US) under an inert nitrogen atmosphere to yield a hydrogel membrane that was approximately 5 μm thick.

Imparting Bioactivity to Hydrogel Electrodes

Before electropolymerization of pyrrole, the hydrogel-modified electrode was immersed and incubated in 5.0 ml of an aqueous Py (0.4 M)/GOx (1 mg/ml) solution prepared in nanopure water for 1 h to ensure equilibrium between the hydrogel film and the electropolymerization solution. Finally, potentiostatic electropolymerization was commenced by the application of 0.75 V vs. Ag/AgCl, 3 M KCl to the Pt W.E leading to polypyrrole formation within the hydrogel membrane [17] and the concomitant immobilization of GOx within the hydrogel. Charge densities tested include 1 and 10 mC/cm^2 applied to the microelectrode. This produced an enzyme-loaded, electroconductive polymer PPy-hydrogel composite. The enzyme electrode, or biotransducer, was subsequently washed with nanopure water and by PBS to remove any unbound GOx from the hydrogel film. Before use, the modified enzyme electrode was over-oxidized by repeatedly cycling the electrode in PBKCl (100 mM, pH 7.0) between 0 and 1.2 V vs. Ag/AgCl, 3 M KCl for 40 cycles.

Characterization of the Enzyme Electrodes

For immobilized enzyme kinetics, biosensor sensitivity, response time and biosensor stability studies, amperometric measurements were performed at 0.4 V vs. Ag/AgCl, 3 M KCl and by sequential injection of appropriate aliquots of glucose solution into 5.0 ml of 100 mM PBKCl (pH 7.0) under continuous stirring to provide for convective transport. Enzyme electrodes were stored in PBKCl solution at 4 °C over a period up to 18 days. The kinetic

parameters were determined using Lineweaver–Burk analysis of biosensor amperometric response every few days during the storage period.

Results and Discussion

Electropolymerization to confer enzyme biospecificity to the Pt-supported hydrogel membrane may be accomplished at different values of electropolymerization charge density. It has previously been established that electropolymerization of Py within electrode-supported hydrogels is initiated at the electrode–hydrogel interface and progresses outwards, being more dense at the electrode–hydrogel interface and progressively less towards the hydrogel–solution interface [23]. The bioactivity of PPy/GOx/poly(HEMA)-based hydrogel was investigated at two charge densities, 1.0 and 10.0 mC/cm^2. Each type of microbiotransducer was investigated by generating a dose–response curve to glucose in aerated buffer solution at RT. Electrodes were then stored in glucose-free buffer solution at 4 °C and periodically analyzed as before by reproducing the dose–response curve. The amperometric enzyme-based biotransducers are Type 1 devices that involve the measurement of the rate of hydrogen peroxide formation that is linked to enzyme activity. The first two reactions [Eqs. 1 and 2] represent the enzyme (GOx) redox reactions, wherein FAD is the oxidized form and $FADH_2$ is the reduced form of the prosthetic group, flavin adenine dinucleotide:

$$\beta\text{–D – glucose} + \text{GOx/FAD} \rightarrow \text{D – glucono–}\delta\text{–lactone} + \text{GOx/FADH}_2 \quad (1)$$

$$\text{GOx/FADH}_2 + \text{O}_2 \rightarrow \text{GOx/FAD} + \text{H}_2\text{O}_2 \quad (2)$$

$$\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}^+ + 2\text{e}^- \quad (3)$$

Figure 1 shows a typical dose–response curve for the amperometric response of a glucose responsive biotransducer interrogated at 0.40 V vs. Ag/AgCl, 3MCl$^-$ at RT. This particular biotransducer was prepared by electropolymerization of Py from an aqueous solution of Py (0.40 M) and GOx (1 mg/ml) in DI-water at RT using a total electropolymerization charge

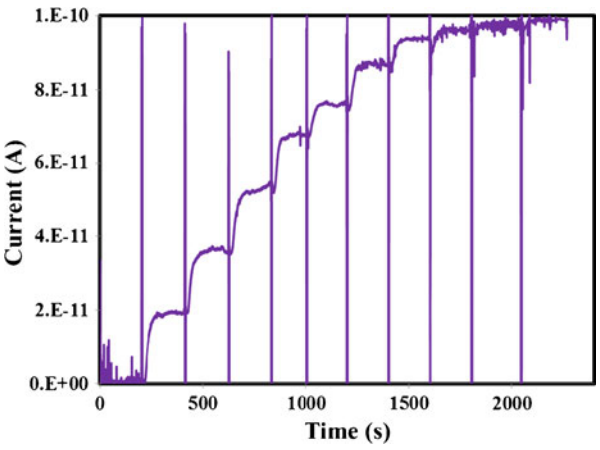

Fig. 1 Dose–response curve for the amperometric response of glucose at a PPy/GOx/Hydrogel‖Acrylate-PEG-APTMS‖Pt biotransducer following an electropolymerization charge density, Q=10 mC/cm^2 and measured at RT after 3 days of storage in PBS 7.4 at 4 °C

density of 10.0 mC/cm^2 and analyzed after 3 days of storage in PBS 7.4 at 4 °C. Figure 2 shows a typical sensitivity plot of the amperometric response of a glucose responsive biotransducer interrogated at 0.40 V vs. Ag/AgCl, 3MCl$^-$ at RT. This particular biotransducer was prepared by electropolymerization of Py from an aqueous solution of Py (0.40 M) and GOx (1 mg/ml) in DI-water at RT using a total electropolymerization charge density of 1.0 mC/cm^2 and analyzed after 11 days of storage in PBS 7.4 at 4 °C. From these plots parameters such as sensitivity, linear dynamic range, response rate, and detection limit may be determined [24].

To study biotransducer stability, biotransducers were stored at 4 °C in glucose-free PBS solution. Figure 3 shows the sensitivity plots of the amperometric response of a glucose responsive biotransducer prepared by electropolymerization of Py using a total electropolymerization charge density of 1.0 mC/cm^2 and analyzed after 11, 15, 16, and 18 days of storage in PBS 7.4 at 4 °C.

Enzyme kinetic parameters were obtained from the characterization of the amperometric enzyme biotransducer. A Lineweaver–Burk analysis using Eq. 4 allows calculation of the apparent enzyme kinetic parameters.

$$\frac{1}{I_{SS}} = \frac{K_M}{I_{max}} \frac{1}{C_S} + \frac{1}{I_{max}} \tag{4}$$

Here I_{SS} is the steady-state current or response after substrate, S, addition, C is the bulk concentration of substrate and I_{max} is the maximum current or response measured under saturated substrate solution. The maximum current, I_{max}, and the Michaelis–Menten constant of the system, K_M (mM), may thus be determined.

Long-Term Stabilization of Enzymes in Electroconductive Polypyrrole-Poly(HEMA) Hydrogels

Michaelis–Menten enzyme parameters for GOx have been well documented and are therefore used to gain insight into factors influencing enzyme activity and biotransducer responsiveness. For amperometric biosensors, the amount of active enzyme immobilized will directly influence sensitivity, maximum current, and apparent enzyme affinity [25]. The apparent Michaelis–Menten constant (K_{Mapp}) for the charge density of the electroconductive hydrogel prepared with an electropolymerization charge of 1.0 mC/cm^2 was found to fluctuate over time, Table 1. For biorecognition membranes prepared with an electropolymerization charge of 10.0 mC/cm^2 the apparent Michaelis–Menten constant (K_{Mapp}) did not

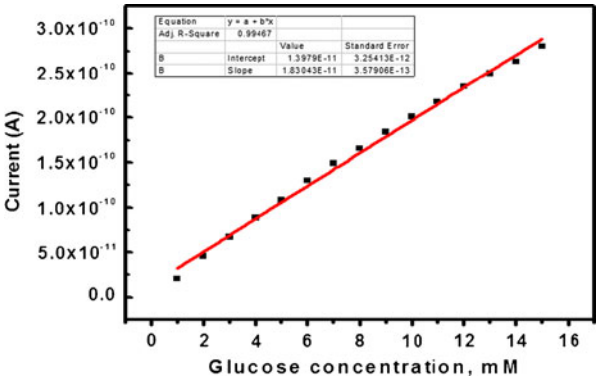

Fig. 2 Sensitivity plot of the amperometric response of glucose at a PPy/GOx/Hydrogel‖Acrylate-PEG-APTMS‖Pt biotransducer following preparation using an electropolymerization charge density, Q=1.0 mC/cm^2 and measured at RT after 3 days of storage in PBS 7.4 at 4 °C

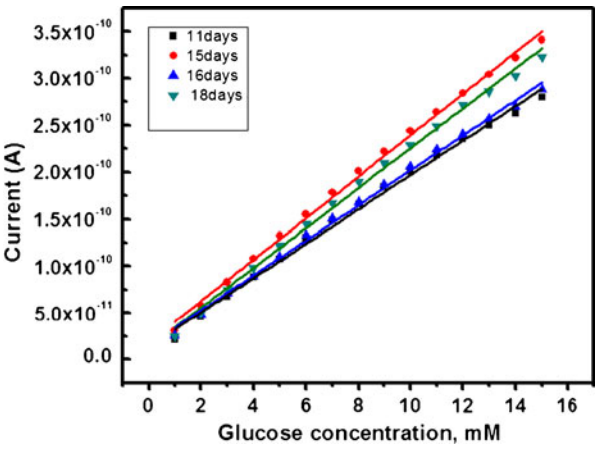

Fig. 3 Sensitivity plots of the amperometric response of glucose at a PPy/GOx/Hydrogel‖Acrylate-PEG-APTMS‖Pt biotransducer following preparation using an electropolymerization charge density, Q=1.0 mC/cm^2 and measured at RT after 11, 15, 16 and 18 days of storage in PBS 7.4 at 4 °C

change as dramatically, Table 2. Enzyme stability and affinity appeared to be maintained considerably higher over time for membranes prepared with 10 mC/cm^2, indicating that the additional polypyrrole introduced more enzyme and aids in maintenance of enzyme activity. Michaelis constants ranging from 2.7–6.1 mM indicate that affinity increases for polypyrrole entrapped GOx compared to native GOx under aerated conditions which typically have K_{Mapp} of approximately 11 mM [26, 27].

It is expected that most of the enzyme molecules that were incorporated through entrapment would somehow have their native conformation and active site perturbed. Changes in protein structure as a result of immobilization may increase or decrease enzyme affinity for substrates. However, it has been shown that substrate specificity of GOx is unaffected by the immobilization through electropolymerization of pyrrole [28]. Polypyrrole has also been seen to have a stabilizing effect on the structure and activity of GOx [29]. Higher charge density entails a thicker layer of polypyrrole grown upon the electrode surface allowing greater immobilization and stabilization of enzyme resulting in more consistent enzyme affinity.

Bare platinum electrodes with pristine electrodeposited polypyrrole and GOx have been shown to approach a diffusion limited response upon reaching a PPy film thickness ≥250 nm [30]. Upon reaching a diffusion limited state, the current response of biosensors become mass transport controlled. This is usually characterized by a decrease in maximum current and sensitivity with increasing film thickness [30]. Assuming a uniform current distribution to the working electrode, potentiostatic growth of polypyrrole films grown at a charge density of 45 mC/cm^2 onto planar platinum substrates will produce films of 0.1 μm thickness

Table 1 Bioanalytical performance and apparent enzyme kinetic parameters of a PPy/GOx/Hydrogel‖Acrylate-PEG-APTMS‖Pt biotransducer following preparation using an electropolymerization charge density, Q=1.0 mC/cm^2 and measured at RT after 7, 10, 11, 15, 16 and 18 days of storage in PBS 7.4 at 4 °C

Days stored	7	10	11	15	16	18
Sensitivity (nA/mM)	11.6	17.5	18.3	22.1	18.6	21.2
Linear dynamic range (mM)	1–8	1–10	1–15	1–15	1–15	1–15
Response time (s)	50	50	50	50	50	50
Apparent Michaelis–Menten constant (mM)	18.6	152	56	33	31.4	104
Maximum current (nA)	0.289	2.90	1.33	1.035	0.84	2.7

Table 2 Bioanalytical performance and apparent enzyme kinetic parameters of a PPy/GOx/Hydrogel‖Acrylate-PEG-APTMS‖Pt biotransducer following preparation using an electropolymerization charge density, Q= 10.0 mC/cm^2 and measured at RT after 3, 4, 7, 8, and 10 days of storage in PBS 7.4 at 4 °C

Days stored	3	4	7	8	10
Sensitivity (pA/mM)	6.4	3.6	8.5	10.6	12.8
Linear dynamic range (mM)	0–7	0–7	1–10	1–10	1–15
Response time (s)	50	50	50	50	50
Apparent Michaelis–Menten constant (mM)	2.7	2.1	4.9	6.0	6.1
Maximum current (pA)	45.5	30	100	147	170

[31]. Thus, the charge densities utilized in this work effectively generate polypyrrole films that are 2.2 and 22 nm in thickness for charge densities of 1.0 and 10.0 mC/cm^2, respectively.

Long-term Effects of Polypyrrole on Amperometric Response

Since the experimental conditions used did not exceed the diffusion limiting polypyrrole film growth thickness, more enzymes were expected to be immobilized using the higher charge density resulting intuitively in an increased maximum current response and sensitivity [30, 31]. Entrapped enzymes have been shown to contribute 90% or more of the amperometric response for PPy electropolymerized sensors compared to the enzymes adsorbed onto the electrode surface [30]. However, the contrary was observed. The lower charge density of 1 mC/cm^2, when compared to 10 mC/cm^2, had twice the sensitivity and a maximum current that was greater by an order of magnitude suggesting that the hydrogel-co-polypyrrole system used in this work quickly reaches mass transport limited kinetics or that enzyme denaturation accompanies higher charge densities for bioimmobilization.

The current response of polypyrrole entrapped GOx sensors is known to be a function of both the film thickness of polypyrrole as well as the kinetics of the electropolymerization process [30]. The poly(HEMA) hydrogel membrane by itself presents an appreciable diffusional barrier [32]. Therefore, addition of the polypyrrole network may increase this diffusional barrier further. Electropolymerization times were relatively long in solutions containing no supporting electrolyte to allow glucose oxidase to serve as the macromolecular counter anion for positively charged polypyrrole networks. This enabled slower network growth of polypyrrole which may have resulted in a more dense co-network and an overall less permeable electroconductive hydrogel.

It is feasible that the polypyrrole network may be continuing to overoxidize even at the mild potential of 0.4 mV vs. Ag/AgCl (3 M KCl) [33]. This would cause significant changes in the structure and chemistry [34] of the interpenetrating networks resulting in additional polypyrrole crosslinking and an increase in rigidity [35] during each interrogation processes requiring over an hour to complete. It is known that the diffusion of hydrogen peroxide through a polypyrrole network is a limiting factor to the magnitude of response for the oxidoreductase enzyme biosensor system used [28]. The experimentally determined diffusion coefficient of oxygen and hydrogen peroxide through over-oxidized polypyrrole film has been shown to be approximately 5.0×10^{-9} cm^2/s [30]. Enzyme efficiency will be highest near the hydrogel–solution interface and decrease closer toward the electrode surface. This would lead to a gradient of hydrogen peroxide production within the hydrogel. The greater diffusional barrier of the 10 mC/cm^2 density biotransducers allows more hydrogen peroxide to diffuse back into the bulk of the solution.

Maturation Phenomena of Biosensors Fabricated by Electrodeposition

A simultaneous increase in sensitivity and dynamic range is desirable for a biosensor system, and both were observed to occur over time at both charge densities studied. Figure 4 shows the progress in the steady-state amperometric response to a 5.0 mM glucose challenge at a PPy/GOx/Hydrogel‖Acrylate-PEG-APTMS‖Pt biotransducer prepared using an electropolymerization charge density, Q=1.0 mC/cm^2, and measured at RT following storage in PBS 7.4 at 4 °C. The trend is a steady increase towards an asymptotic limit. This phenomenon can be considered a maturation process for the biosensor system. The linear dynamic range increased and leveled off at 1–15 mM. The current generated by the biosensor reflects the rate at which glucose oxidase enzymes are capable of turning over glucose into hydrogen peroxide. The observed increase in sensitivity and maximum current suggests that the biotransducer's turnover rate for glucose increases over time. Stability of the response time at 50 s is evidence that there is little to no disbondment between the hydrogel and electrode surface as diffusion through the hydrogel would be compromised. Similar biosensors, with enzymes directly cross-linked within the hydrogel, without polypyrrole and stored desiccated in the absence of buffer, have been shown to retain >80% of their initial response during the first 3 weeks of storage and up to 70% of their initial response after 8 months of storage [9]. Therefore, it was expected that the biotransducers would lose sensitivity to glucose. Further prospective evidence is still necessary to understand the reasons for the maturation process of biosensor sensitivity.

Conclusions

Following electropolymerization at low charge density, the partitioned and adsorbed enzymes within the hydrogel may become trapped, have more enzyme active sites available for reaction, less diffusional constraints, but will be more unstable. Electrodes of 10 mC/cm^2 gels were capable of entrapping enzymes within the hydrogel and polypyrrole, maintain their affinity, but also lead to an increase in diffusional barriers for enzyme substrate and products. A tradeoff between enzyme stability over time and magnitude of current response over time appears to be necessary. It is concluded that enzyme incorporation into hydrogels via electropolymerization of polypyrrole is an effective means of imparting bioactivity to a transducer, but the biotransducer becomes more sensitive to the mass transport limitations imparted by the over-oxidized polypyrrole.

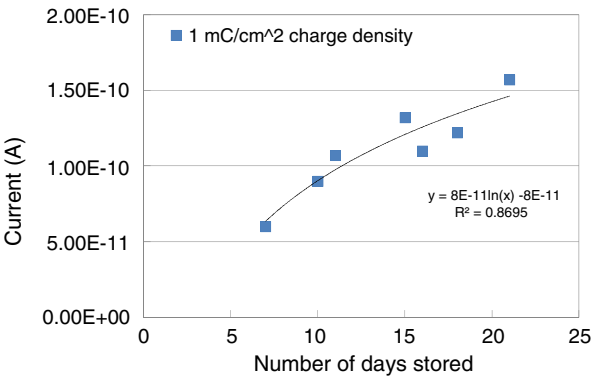

Fig. 4 Progress in the steady-state amperometric response to a 5.0 mM glucose challenge at a PPy/GOx/Hydrogel‖Acrylate-PEG-APTMS‖Pt biotransducer prepared using an electropolymerization charge density, Q=1.0 mC/cm^2, and measured at RT following storage in PBS 7.4 at 4 °C

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References

1. Guiseppi-Elie, A., et al. (2011). The effect of temperature on the impedimetric response of bioreceptor hosting hydrogels. *Biosensors and Bioelectronics, 26*(5), 2275–2280.
2. Abraham, S., et al. (2005). Molecularly engineered p(HEMA)-based hydrogels for implant biochip biocompatibility. *Biomaterials, 26*, 4767–4778.
3. Gawel, K., et al. (2010). Responsive hydrogels for label-free signal transduction within biosensors. *Sensors, 10*(5), 4381–4409.
4. Guiseppi-Elie, A. (2010). Electroconductive hydrogels: synthesis, characterization and biomedical applications. *Biomaterials, 31*(10), 2701–2716.
5. Joseph, H., Owino, O., et al. (2008). Synthesis and characterization of poly (2-hydroxyethyl methacrylate)-polyaniline based hydrogel composites. *Reactive and Functional Polymers, 68*(8), 1239–1244.
6. Justin, G., & Guiseppi-Elie, A. (2010). An electroconductive blend of p(HEMA-*co*-PEGMA-*co*-HMMA-*co*-SPMA) hydrogels and p(Py-co-PyBA): in vitro biocompatibility. *Journal of Bioactive and Compatible Polymers, 25*(2), 121–140.
7. Cui, X., et al. (2003). In vivo studies of polypyrrole/peptide coated neural probes. *Biomaterials, 24*(5), 777–787.
8. Brahim, S., Narinesingh, D., & Guiseppi-Elie, A. (2002). Interferent suppression using a novel polypyrrole-containing hydrogel in amperometric enzyme biosensors. *Electroanalysis, 14*(9), 627–633.
9. Brahim, S., Narinesingh, D., & Guiseppi-Elie, A. (2002). Polypyrrole-hydrogel composites for the construction of clinically important biosensors. *Biosensors and Bioelectronics, 17*(1–2), 53–59.
10. Guiseppi-Elie, A. (2010). An implantable biochip to influence patient outcomes following trauma-induced hemorrhage. *J Anal and Bioanal Chem, 339*(1), 403–419.
11. Guiseppi-Elie, A., Brahim, S., & Wilson, A. (2006). Biosensors based on electrically conducting polymers. In T. Skotheim & J. R. Reynolds (Eds.), *Handbook of conducting polymers: conjugated polymer processing and applications* (3rd ed., pp. 435–479). New York: Marcel Dekker. Biosensors based on electrically conducting polymers.
12. Brahim, S., et al. (2003). Chemical and biological sensors based on impedimetric detection using conductive polymers. *Microchimica Acta, 143*, 123–137.
13. Yamato, H., Ohwa, M., & Wernet, W. (1995). Stability of polypyrrole and poly(3,4-ethylenedioxythiophene) for biosensor application. *Journal of Electroanalytical Chemistry, 397*(1), 163–170.
14. Foulds, N. A., & Lowe, C. R. (1986). Immobilisation of glucose oxidase in polypyrrole and its application in amperometric glucose sensors. *J Chem SocFaraday Trans, 82*, 1259–1264.
15. Brahim, S., Narinesingh, D., & Guiseppi-Elie, A. (2002). Bio-smart materials: kinetics of immobilized enzymes in p(HEMA)/p(pyrrole) hydrogels in amperometric biosensors. *Macromolecular Symposia, 186*, 63–73.
16. Duffitt, G. L., & Pickup, P. G. (1991). Permselectivity of polypyrrole in acetonitrile. *Journal of Physical Chemistry, 95*(24), 9634–9635.
17. Kotanen, C., Karunwi, O., & Guisepp-Elie, A. (2010). Physiological status monitoring for glucose and lactate during the onset of hemorrhagic shock. *American Society of Gravitational and Space Biology Bulletin, 23*(2), 55–63.
18. Rahman, A. R., Justin, G., & Guiseppi-Elie, A. (2009). Towards an implantable biochip for glucose and lactate monitoring using micro-disc electrode arrays (MDEAs). *Biomedical Microdevices; BioMEMS and Biomedical NanoTechnology Biomedical Microdevices, 11*(1), 75–85.
19. Guiseppi-Elie, A. and N.F. Sheppard Jr. *Conferring biospecificity to electroconductive polymer-based biosensor devices*. in *ACS Northeast Regional Meeting (NERM)*. 1995. Rochester, NY.
20. Guiseppi-Elie, A. (2011). An implantable biochip to influence patient outcomes following trauma-induced hemorrhage. *Analytical and Bioanalytical Chemistry, 399*(1), 403–419.
21. Brahim, S., & Guiseppi-Elie, A. (2005). Electroconductive hydrogels: electrical and electrochemical properties of polypyrrole-poly(HEMA) composites. *Electroanalysis, 17*(7), 556–570.
22. Boztas, A. O., & Guiseppi-Elie, A. (2009). Immobilization and release of the redox mediator ferrocene monocarboxylic acid from within cross-linked p(HEMA-*co*-PEGMA-*co*-HMMA) hydrogels. *Biomacromolecules, 10*(8), 2135–2143.

23. Justin, G., & Guiseppi-Elie, A. (2009). Characterization of electroconductive blends of p(HEMA-*co*-PEGMA-*co*-HMMA-co-SPMA) hydrogels and p(Py-*co*-PyBA). *Biomacromolecules, 10*(9), 2539–2549.
24. Theâvenot, D. R., et al. (1999). Electrochemical biosensors: recommended definitions and classification. *Pure and Applied Chemistry, 71*(12), 2333–2348.
25. Guerrieri, A., et al. (1998). Electrosynthesized non-conducting polymers as permselective membranes in amperometric enzyme electrodes: a glucose biosensor based on a co-crosslinked glucose oxidase/over-oxidized polypyrrole bilayer. *Biosensors and Bioelectronics, 13*(1), 103–112.
26. Wolowacz, S. E., Yon Hin, B. F. Y., & Lowe, C. R. (1992). Covalent electropolymerization of glucose oxidase in polypyrrole. *Analytical Chemistry, 64*(14), 1541–1545.
27. Gao, F., Courjean, O., & Mano, N. (2009). An improved glucose/O_2 membrane-less biofuel cell through glucose oxidase purification. *Biosensors and Bioelectronics, 25*(2), 356–361.
28. Fortier, G., Brassard, E., & Bélanger, D. (1990). Optimization of a polypyrrole glucose oxidase biosensor. *Biosensors and Bioelectronics, 5*(6), 473–490.
29. Fortier, G., & Bélanger, D. (1991). Characterization of the biochemical behavior of glucose oxidase entrapped in a polypyrrole film. *Biotechnology and Bioengineering, 37*(9), 854–858.
30. Gros, P., & Bergel, A. (1995). Improved model of a polypyrrole glucose oxidase modified electrode. *Journal of Electroanalytical Chemistry, 386*(1–2), 65–73.
31. Palmisano, F., et al. (1995). Correlation between permselectivity and chemical structure of overoxidized polypyrrole membranes used in electroproduced enzyme biosensors. *Analytical Chemistry, 67*(13), 2207–2211.
32. Justin, G., et al. (2009). Biomimetic hydrogels for biosensor implant biocompatibility: electrochemical characterization using micro-disc electrode arrays (MDEAs). *Biomedical Microdevices: BioMEMS and Biomedical NanoTechnology Biomedical Microdevices, 11*(1), 103–115.
33. Rodríguez, I., Scharifker, B. R., & Mostany, J. (2000). In situ FTIR study of redox and overoxidation processes in polypyrrole films. *Journal of Electroanalytical Chemistry, 491*(1–2), 117–125.
34. Witkowski, A., Freund, M. S., & Brajter-Toth, A. (1991). Effect of electrode substrate on the morphology and selectivity of overoxidized polypyrrole films. *Analytical Chemistry, 63*(6), 622–626.
35. Otero, T. F., Márquez, M., & Suárez, I. J. (2004). Polypyrrole: diffusion coefficients and degradation by overoxidation. *The Journal of Physical Chemistry. B, 108*(39), 15429–15433.