Letter

Composite hydrogels containing bioactive microreactors for optical enzymatic lactate sensing

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Composite hydrogels containing bioactive microreactors for optical enzymatic lactate sensing

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KEYWORDS

Biosensor, lactate sensing, composite hydrogel, layer-by-layer self-assembly, alginate, phosphorescence.

ABSTRACT

Continuously monitoring specific biomarkers offer a promising method to interrogate disease status and progression. In this work we have demonstrated a composite hydrogel-based sensing platform that may be used for optical detection of lactate. The sensor design consists of micro-sized enzymatic sensors that are embedded in an outer hydrogel matrix. In these engineered microdomains, encapsulated lactate oxidase serves as the bioactive component, phosphorescent metalloporphyrin acts as the optical transducer, and polyelectrolyte multilayers coated on the enzymatic micro-sensors controls the permeation of lactate into the micro-sensors. The response of the composite hydrogel-based lactate sensors was characterized by subjecting them to lactate concentration challenges at low physiological oxygen levels. The analytical range and the mean sensitivity were determined to be 9.2 ± 0.83 mg/dL and 11 ± 0.90 % dL mg^{-1} respectively. Repeated cyclic exposure to high levels of lactate revealed that these sensors were extremely stable, with no significant loss in sensor response after 20 cycles. These preliminary results support the premise that these composite hydrogels are capable of continuous lactate tracking and have potential for use as fully implantable optical lactate sensors.

Biosensors employing enzymes as the molecular recognition component have found widespread use in industrial chemical, biotechnology, and clinical chemistry applications as key elements in process control and medical diagnostics.[1] Enzymatic biosensors commonly contain an oxidoreductase enzyme (oxidase or dehydrogenase) that catalyzes the enzymatic utilization of the substrate accompanied by a specific product formation. Substrate depletion and/or byproduct formation is then monitored optically or electrochemically.[1] Such biosensors are "flux-dependent" systems and operate using the principle of a reaction that is rate-controlled by the diffusion of substrate. Hence, a substrate-reactive enzyme module and a diffusion-limiting coating are both key components of an enzymatic biosensor. Enzymatic biosensors based on these concepts have been developed for sensing various analytes (*e.g.* cholesterol, lactate, urea, ethanol, ascorbic acid, bilirubin, choline, glutamine, uric acid, and glucose).[1]

Lactate, a primary byproduct of anaerobic metabolism is an analyte of interest due to its relevance in the food industry, sports medicine, and critical care.[2-4] Monitoring blood lactate concentrations in patients with severe ketoacidosis is essential to prevent muscle damage and potential heart attack.[5] Additionally, lactate tracking can be used to identify patients requiring resuscitative care in the event of significant blood loss.[6] Continuous lactate monitoring is also beneficial during exercise and fitness regimes in order to prevent excessive lactate buildup.[7-8]

As noted above, enzymatic biosensors employ diffusion-limiting coatings to restrict the amount of substrate entering the enzyme module per unit time, effectively making the system substrate-transport-limited rather than reaction-kinetics-limited.[9] In enzymatic lactate sensors, LOx (lactate oxidase) catalyzes the oxidation of lactate in the presence of molecular oxygen, ultimately producing pyruvate (lactate + O_2 + lactate oxidase + H_2O $\rightarrow$ pyruvate + H_2O_2). If the enzyme is in excess and the reaction is not limited by oxygen supply, the reaction proceeds at a

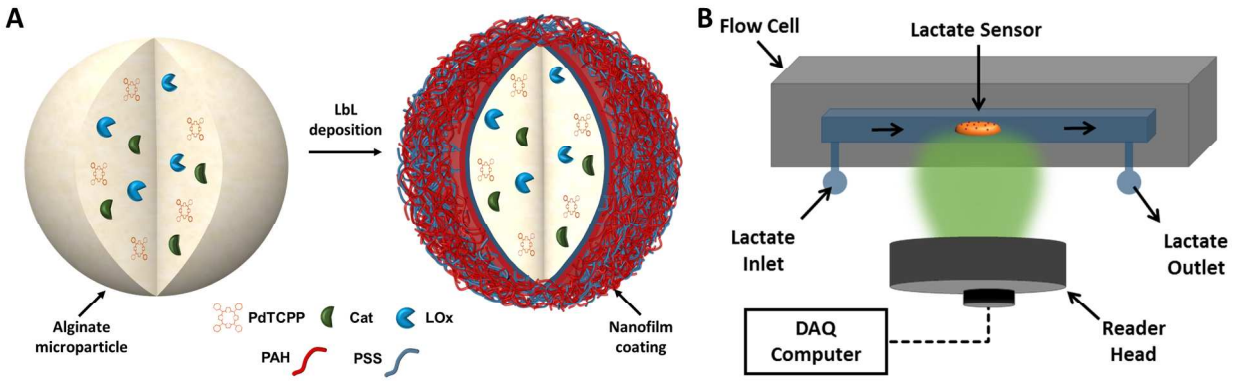
rate proportional to the amount of lactate oxidized. As a consequence, the corresponding decrease in molecular oxygen and the release of hydrogen peroxide are also lactate-dependent. Thus, measuring fluctuations in molecular oxygen or hydrogen peroxide enables the indirect measurement of lactate concentrations.

Ideally, the oxygen consumed in these enzymatic reactions should be rapidly replenished in order to maintain a lactate diffusion-limited sensor response.[10] However, maintaining a constant local oxygen concentration becomes a challenge in certain applications when oxygen is in short supply, especially for implanted sensors. Normal tissue oxygen concentrations are substantially lower than to ambient oxygen levels; worse, most implants reside within inflamed or scarred tissue, where oxygen supply decreases by ~ 75% over a period of three weeks after implantation.[11] These conditions demand special attention to sensor design and testing.

While there are commercial blood lactate sensors and studies on lactate biosensors have been may be found in the literature, to our knowledge previous work has not focused on developing designs suitable for full implantation as well as adjustment for operation in extreme conditions. Nearly all enzymatic lactate sensors described to date have been based on planar or cylindrical transducers (*e.g.* wires, optical fibers, patterned electrodes, or waveguides) with a coating applied to the outer surface to control analyte transport.[12-15] These devices require physical connections for measurements and have a narrow range of possible mechanical properties. These constraints limit their potential use in many applications, particularly for use as transcutaneous sensing devices where chronic inflammation leads to eventual sensor failure within a few days.[16] Alternatively, optical sensing technologies based on soft hydrogel based materials may be used to circumvent these drawbacks; optical sensor interrogation obviates the need for a physical connection and the hydrogel matrix provides a low-fouling, biocompatible surface.[17-18]

Herein, we describe a lactate sensing system relying on the combined effect of a population of microdomains dispersed within a hydrogel matrix to generate a measurable change in an optical property (phosphorescence). In this format the microdomains contain bioactive lactate oxidase along with an oxygen-sensitive reporter dye. Lactate oxidase catalyzes lactate, depleting local oxygen levels that in turn increases the phosphorescent intensity and lifetime of the reporter dye; monitoring the change in lifetime enables the indirect detection of lactate. The sensing microdomains are further lined by polyelectrolyte multilayers (PEMs), a surface coating that provides a means to control local diffusion properties and to alter sensor response. Optical sensors based on these active domains embedded within a soft hydrogel are suitable for aqueous analysis, including potential use as implantable devices if an appropriately biocompatible matrix is employed. The modular design allows independent control over sensor response and biocompatibility; by altering the permeability of analyte across the PEMs the sensor response can be adjusted [19-20] and the biocompatibility can be manipulated by controlling the properties of the outer hydrogel matrix.

We hypothesized that PEM lined microreactors containing enzyme and phosphorescence reporter could be entrapped in an outer hydrogel matrix to be used as optical lactate biosensors with fully-reversible response at physiologically relevant oxygen conditions. To our knowledge, lactate-responsive materials of this nature have not been demonstrated before. Therefore, we fabricated nanofilm-bounded LOx, catalase, and phosphor-containing microreactors using alginate microparticle templates, which were ultimately dispersed in an alginate matrix to be used as composite hydrogel based lactate sensors. We evaluated the response of these sensors to lactate at ambient and low oxygen concentrations and further examined their stability during repeated cyclic lactate exposure.



Scheme 1. Representations of **(A)** lactate-responsive alginate microparticles with multilayer nanofilm coating and **(B)** flow-through system and reader head used to test response of hydrogels with embedded sensing microdomains.

RESULTS AND DISCUSSION

Nanofilms (10 bilayers of PAH/PSS) were deposited on microparticles containing LOx/Cat/PdTCPP using the LbL approach that involves coating the microparticles with oppositely charged polyelectrolyte layers (Scheme 1A). The zeta potential of the microparticles was measured after deposition of each PEM to confirm successful surface charge reversal (Figure 1); as expected PAH-terminated coatings exhibited a positive zeta potential (49.7 ± 5.20 mV) whereas coatings with PSS outer layers resulted in a negative value (-20.6 ± 2.40 mV). The average potentials of the same terminal layers exhibit some fluctuations as the number of layers increase; however, the repeated measurements reveal very consistent behavior between batches, indicating the results are reliable. After deposition of 10 bilayers, the nanofilm-coated alginate microparticles were used to fabricate lactate sensing hydrogels. SEM images revealed the

morphology of both the nanofilm coated microparticles (Figure 2A) and the composite lactate sensing hydrogels (Figure 2B). The $[PSS/PAH]_{10}$ coated alginate microparticles were found to be spherical and covered by a "fuzzy" outer layer, characteristic of PEM-coated colloidal particles.[21] From the SEM images of the hydrogel, one may observe multiple microparticles with a wrinkled architecture entrapped within an outer hydrogel matrix. DIC images were used to determine the mean diameter of the $[PSS/PAH]_{10}$ coated microparticles as 10.9 ± 0.460 μm. From the confocal image (Figure 2C) it can be clearly seen that the phosphorescent PdTCPP dye (the hydrogel's excitation and emission spectra are depicted in Figure S2) is restricted within the PEM-lined alginate microparticles. This was anticipated, as the nanofilm coating localizes the macromolecules (LOx, Cat, and PdTCPP) within the spherical microdomains. Next we verified the oxygen-sensitive behavior of the reporter dye; in the presence of molecular oxygen, phosphorescent porphyrin dyes are quenched by collisions with oxygen, resulting in decreased phosphorescence intensity and decay lifetimes. Fluorescence intensity ratiometric imaging (Figure 2D) was used to confirm that the lactate sensing bioreactors were sensitive to changes in oxygen. The intensity ratio >1 for reduced oxygen conditions ratifies that the phosphorescence of PdTCPP in the microreactors increases with a decrease in oxygen level.

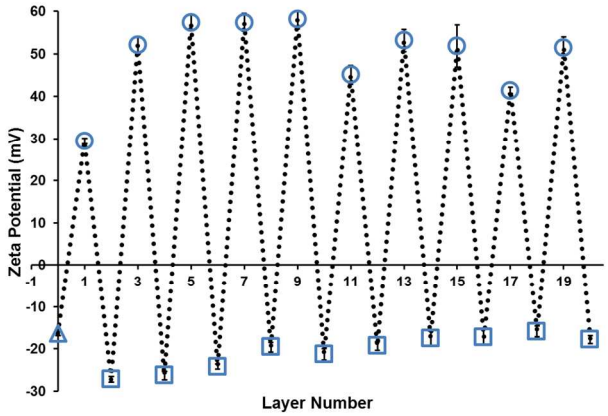


Figure 1. Change in zeta potential with increase in the number of polyelectrolyte layers coated on alginate microparticles. Δ = bare alginate microparticles, ○ = PAH and □ = PSS. Error bars represent 95% confidence intervals for measurements performed on three separate batches.

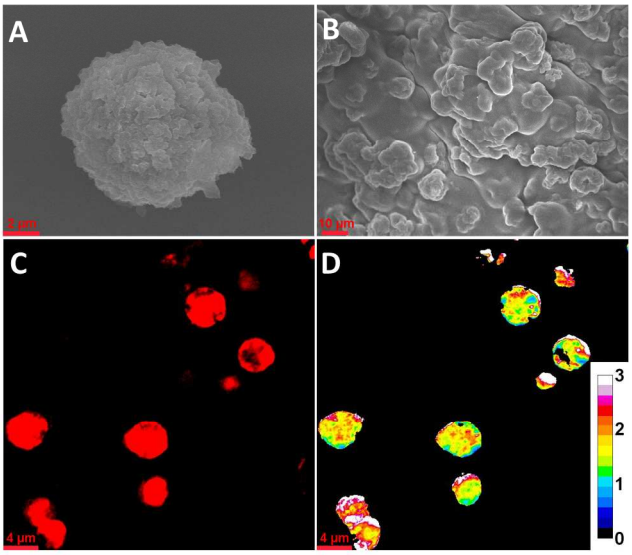


Figure 2. Scanning electron micrographs of **(A)** alginate microparticles with nanofilm coatings, **(B)** alginate hydrogel containing dispersed bioactive PEM lined alginate microparticles. Fluorescence microscopy images of alginate hydrogel containing dispersed bioactive PEM lined alginate microparticles at ambient oxygen **(C)** and ratiometric intensity image **(D)** under reduced oxygen to ambient oxygen.

To quantitatively evaluate the response, the composite hydrogels were exposed to varying concentrations of oxygen in the flow-through system (Scheme 1B). As can be seen from Figure S3, the phosphorescence lifetime response of the hydrogels increases with a decrease in oxygen. In order to construct a Stern-Volmer plot, τ_0/τ (where τ_0 is the unquenched lifetime) was plotted against oxygen (Figure S3 inset); using the Stern Volmer equation $\tau_0/\tau = 1+K_{SV}[O_2]$, the mean K_{SV} was calculated to be 0.042 ± 0.008 μM^{-1}. This result is similar to previously-reported values.[22] It should be noted that at higher oxygen concentrations there is a decrease in linearity that may be attributed to oxygen's unequal accessibility to the dye molecules.[23]

Having established that the LOx/Cat/PdTCPP loaded microreactors are sensitive to oxygen, the response of the hydrogels to lactate was examined more thoroughly. We hypothesized that

composite hydrogels containing embedded LOx/Cat/PdTCPP microdomains can function as reversible lactate biosensors using the principles outlined in the introduction: lactate is oxidized as it enters the bioactive domains of the alginate microparticles, which is accompanied with a corresponding reduction in local oxygen that may then be optically monitored.

Interestingly, the hydrogel-based sensors registered no significant change (p>0.01) in lifetime response (Figure 3) with increasing lactate concentrations at ambient oxygen (~ 21%). This suggested that the lactate flux was very low relative to the oxygen flux under the conditions of testing. Investigating the transport of lactate across planar $[PSS/PAH]_{10}$ nanofilms deposited on porous substrates (Figure S4) revealed that 10 bilayers of PSS/PAH were readily permeable to lactate with a permeation rate (dC/dt) of 0.04 $g.L^{-1}.h^{-1}$. Thus, the insignificant change in lifetime response was observed even though lactate is readily available to LOx contained in the microreactors. This lack of response may be ascribed to a negligible change in oxygen in the presence of lactate, coupled with the lower sensitivity of the phosphorescent oxygen probe at higher ambient oxygen concentrations.

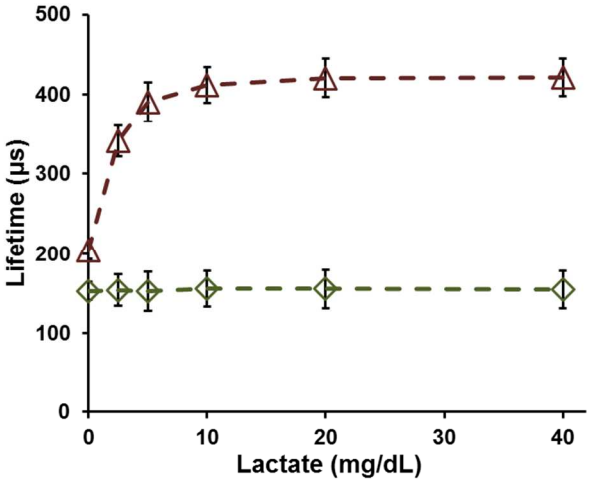


Figure 3. Response of hydrogels with embedded nanofilm-coated microparticles. Plot of lifetimes versus lactate concentration at ambient (green ◊) and low oxygen (maroon Δ) conditions. Error bars represent 95% confidence intervals for three separate batches of bioactive microparticle containing hydrogels.

As noted above, it is more appropriate to characterize sensors that are intended for implantation under low-oxygen conditions, because tissue oxygen level is ~75% lower than ambient oxygen. [11] Therefore, the same set of measurements was performed while the oxygen concentration was reduced to a more physiologically-relevant oxygen (~ 5.5%). From the results reported in Figure 3, in contrast to the response at ambient oxygen, it can be seen that the hydrogel sensors are highly sensitive when exposed to lactate at low dissolved oxygen conditions. The response profile of the sensor was fit to a function of the form $f(x) = a / [\, b+e^{-cx} \,]$ with R^2=0.99, which was used to calculate the sensor parameters. Using the 3-σ method, the *LOD*, *MDLC*, and analytical range (*R*) were calculated as 0.053 ± 0.043 mg/dL, 9.3 ± 0.81 mg/dL and 9.2 ± 0.83 mg/dL, respectively. Additionally, the mean sensor sensitivity was

estimated to be 11 ± 0.90 % dL mg^{-1} over the analytical range. It is important to recognize that this response matches very well to the required range of detection of physiological levels (4.50-13.5 mg/dL) of lactate.[24] These findings demonstrate the basic feasibility of such lactate-sensing microparticles immobilized in a hydrogel matrix for potential application as implantable hydrogel-based biosensors.

Finally, reversibility and stability of the sensors were evaluated by alternately exposing the sensors to high (40 mg/dL) and low (0 mg/dL) levels of lactate. Enzymatic sensors that produce hydrogen peroxide as a by-product are prone to peroxide-mediated enzyme denaturation, which ultimately leads to sensor failure over time.[25] However, the co-immobilization of catalase in the microparticles engenders the catalytic degradation of peroxide which was expected to enhance the stability of the lactate sensors. From Figure 4, it is evident that the baseline lifetime response at 0 mg/dL lactate and the sensor response at 40 mg/dL showed no significant difference ($p > 0.01$) even after 20 cycles (where each cycle was completed in ~ 3 h) of repeated lactate exposure, corroborating the highly stable and reversible nature of the biosensors; using linear regression and extrapolating the average lifetime response at 40 mg/dL it was estimated that the sensor would lose 50% of its optical signal at cycle number 1729.

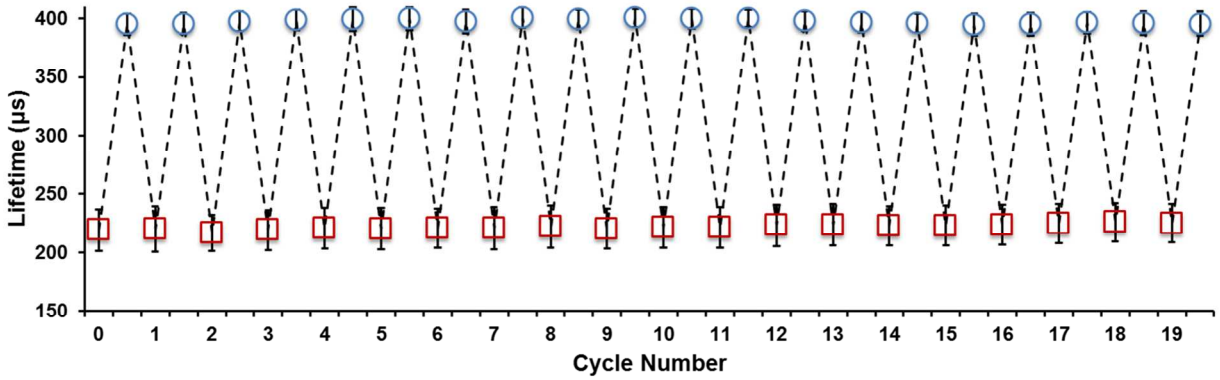


Figure 4. Cyclic testing of lactate sensors when alternately exposed to 0 mg/dL (red □) and 40 mg/dL (blue ○) lactate at low oxygen. Error bars represent 95% confidence intervals for three separate batches of bioactive microparticle containing hydrogels.

CONCLUSION

This work has shown the ability to use composite hydrogels as reversible and stable optical lactate sensors. These hydrogels are embedded with PEM lined microparticles that contain encapsulated enzyme and oxygen sensitive porphyrin dye, which together comprise a fully-enabled bioreactor and "reporter" system with diffusion control at the surface. The LbL nanofilms were able to restrict the sensing components inside the microdomains while allowing the free transport of oxygen and lactate for successful lactate sensing. We demonstrated that these sensors were highly sensitive (11 ± 0.90 % dL mg^{-1}) in the range 0.05 – 9.3 mg/dL when operated at physiological (low) oxygen levels, which is necessary for *in vivo* sensing. Additionally, these sensors exhibited extraordinary stability when repeatedly exposed to high lactate levels, further supporting the potential for long term *in vivo* use. The high degree of stability is ascribed to enhanced LOx stability, which is believed to result from: (1) the presence

of co-immobilized Cat that removes detrimental H_2O_2 and (2) stabilization of LOx's tertiary structure due to interaction with the surrounding polymer matrix.[26] In principle, this composite hydrogel sensor platform can be readily modified to sense other chronic disease biomarkers (*e.g.* glutamate, uric acid) by switching the oxidoreductase enzyme and adjusting the nanofilm properties as needed to control substrate flux. In future work, similar sensors will be implanted in animal models to evaluate *in vivo* sensor response towards potential human deployment.

ASSCOCIATED CONTENT

Supporting Information

The supporting information is available free of charge on the ACS Publications website at DOI:

Experimental section, Lactate permeation across nanofilm data, Stern-Volmer plot (lifetime vs. oxygen concentration), excitation-emission spectra of the lactate sensing composite hydrogel.

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Notes

All authors have approved the final version of this manuscript, and declare no competing financial interest. A.B and L.B. contributed equally.

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