Dual mode detection of heavy metal pollutants: A real-time biosensing method

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Abstract

Objectives: The overall goal of this project is to develop a generic integrated biosensing platform for the detection of heavy metal pollutants in aqueous solutions. We plan to design and fabricate a self-reporting lab-on-chip device, based on dual-detection-modes (optical and electrochemical) that evaluates in real-time the presence of trace levels of metallic pollutants and the overall safety of water samples. *To achieve this goal, our specific aims are:*

1. *Construct the biosensing platform.*
2. *Develop biosensing schemes for heavy metals detection.*
3. *Perform limited testing with simulated water samples demonstrating detection levels at improved sensitivity (lower than the current 60-120 ppb).*

Background to the topic

The proposed research is designed as a response to the important challenge of monitoring heavy metal pollutants in the environment by developing an integrated single-device architecture for on-site detection. Heavy metals are one of the most serious environmental pollution problems of our time, specifically in developing countries, owing to its widespread in nature and high toxicity to living organisms. Long-term exposure causes serious physiological implications, e.g., severe neurological degenerative conditions, heart disease, stillbirth, and cancer, requiring frequent determination in water, soil, agricultural and food samples. Traditional methods of detection, based on laboratory techniques, possess major on-site limitation revealing their inadequacy for real-time analysis. Thus, an early and rapid detection of heavy metals followed by corrective and/or prevention actions can lead to reduction of the overall polluting impact to safeguard public health.

Major conclusions, solutions, achievements

A biosensing platform dedicated for specific and selective monitoring of target analytes was successfully fabricated using a simple and portable experimental setup. We exploit the specificity of several bioreceptors for identifying metallic pollutants, thus the biosensing scheme was modified accordingly. Two target molecules (copper and lead) in simulated water conditions were analyzed by the fabricated transducer. In terms of limit of detection and increased sensitivity, we adapted biochemical reaction (enzymatic reaction product) for signal amplification of lead ions, while specific adsorption was utilized for copper sensing. The overall performance depicts specific recognition of target molecules below the sensitivity levels set by the funding committee (see **aim 3**, lower than 60-120 ppb), presenting detection limits of 54 and 10 ppb for copper and lead, respectively. Additionally, the applicability of the sensor in real-water samples spiked with different copper concentrations was also shown, presenting sufficient recovery rates (85-117%). We also investigated electrochemical biosensors. We evaluated microbes from heavy metal polluted environments (i.e. *Pseudomonas veronii* 2E, an autochthonous bacterial isolate belonging to a Cr-contaminated environment in Buenos Aires (Argentina)) to find bioelectrocatalysts that could be used for electrochemical heavy metal biosensing. We used a combination of electroanalysis and bioinformatics to determine relevant microbes and then evaluated their ability to be utilized as biosensors for heavy metal pollution. For instance, *Pseudomonas veronii* 2E was utilized for chromium detection in simulated wastewater to detection limits of 2.4ppm. This detection limit is a result of slow transport of analyte across the microbial cell membrane.

Implications both scientific and agricultural

The suggested feasibility research presents a multidisciplinary approach for the development of new class of biosensing architecture for analytical biosensing applications. Continuation project should address the realization of the research goals providing profound advantages in terms of speed, sensitivity, cost of measurement, label-free, multicomponent data collection, as well as portability of the system for on-site detection.

Contribution of the collaboration

The two research teams worked on a common goal of developing a generic integrated biosensing platform for the detection of heavy metal pollutants in aqueous solutions. Each team has focused on prototype construction, optimization and characterization to fulfill the most important aim of this project, which was set by the funding committee – **aim 3** *improved sensitivity (lower than the current 60-120 ppb)*. The Israeli team focused on the optical platform, while the US team focused on the electrochemical transduction, both concentrated at improved sensitivity levels. The Israeli graduate student visit to Minter's laboratory was omitted due to time constrain of the project and change from full project duration to a feasibility examination, resulting in overall aims adjustment.

*Achievements:***Significance of main scientific achievements and innovation**

The proposed feasibility research is designed as a response to the important challenge of monitoring heavy metal pollutants in the environment by developing an integrated single-device architecture for on-site detection, specifically three model ions. Thus, we have aimed to develop a self-reporting lab-on-chip device, based on dual-detection-modes (optical and electrochemical) that evaluates in real-time the presence of trace levels of metallic pollutants and the overall safety of water samples. The first stage of the project was to fabricate a biosensing platform individually and then merge both components to a single-device. Herein, porous Si optical biosensors were constructed by electrochemical anodization of Si wafer, producing two distinct optical nanostructures, monolayer and microcavity. The latter provides sharp spectral features for improved spectral sensitivity to fulfill the requirement of detecting heavy metals concentrations as low as 10 ppb. Two biosensing approaches were investigated: synthetic residues (branched polyethylenimine) interaction with copper ions and enzymatic reaction amplification of DNAzyme cleavage in the presence of lead contaminants. Both approaches were successfully fabricated and fully characterized. Next, the selectivity of the platforms for multi-analytes was monitored through optical experiments. Changes in the reflectivity spectrum are monitored in real-time by acquisition of the reflectivity spectra of the porous thin-film upon target capturing. Wavelength shift or amplitude intensity were utilized for each sensing procedure. The sensitivity was verified upon introduction of different water samples spiked with metallic pollutants at various concentrations. The synthetic interaction presented dynamic linear range of the platform between 200 to 2000 ppb, while the calculated limit of detection is 54 ppb. The DNAzyme concept was examined at concentrations up to 50 ppb of lead, while sensing concentrations as low as 10 ppb within 50 min of bioassay were obtained. This result further strengthens the applicability of the presented biosensing concept for practical applications, as most heavy metals assays require detection limits of a few ppb in real water samples. Continuation project should be focused at increasing the sensitivity of the platform to higher sensitivity values. The practical aspect of the resulting optical platform was also investigated in three water samples presenting satisfactory recovery values. In contrast to laboratory-based techniques, the developed platform can be easily adapted for on-field conditions without hindering optical resolution. Little is known about the adaptation strategies adopted by microorganisms to cope with chemical changes happening in the environment, and the effects on microbial electrochemical technologies.

Therefore, we combined bioinformatic analysis with electrochemical analysis to understand adaptation genetics and how that can alter sensing of chemical changes in the microenvironment. For instance, bioinformatics analysis revealed a metabolism shift in *Rhodobacter capsulatus* resulting from salt stress, with changes in gene expression allowing accumulation of compatible solutes to balance osmotic pressure, together with the up-regulation of the nitrogen fixation cycle, an electron sink of the photosynthetic electron transfer chain. Using the transcriptome evidence of hindered electron transfer in the photosynthetic electron transport chain induced by adaption to salinity, increased understanding of bioelectrocatalysis under chemical stress was achieved. This work provides a proof of concept for the combination of bioinformatics and electrochemical tools to investigate microbial electrochemical systems, opening exciting future research opportunities.

A portable self-powered microbial electrochemical biosensor enabling online monitoring of Cr(VI) was accomplished. The biosensor employed a bio-inspired redox mediating system to allow extracellular electron transfer between a bacterial isolate from chromium contaminated environments and the electrode, providing a clear response to Cr(VI) presence. The biosensor showed good linearity ($R^2 = 0.983$) and a limit of detection of $2.4 \text{ mg L}^{-1} \text{ Cr(VI)}$, with a sensitivity of $0.31 \pm 0.02 \text{ } \mu\text{A cm}^{-2} \text{ mgCr(VI)}^{-1} \text{ L}$. This microbial bioanode architecture enhanced biosensor performance thanks to the enhanced “electrical wiring” between biological entities and the abiotic electrode surface. This approach could be easily implemented in engineered electrode surfaces, such as paper-based multi-anodes with the isolated oxidoreductases from the microorganism, further improving biosensor response. Unfortunately, with the short time frame of the one year project, it was not possible to move to the isolated and purified oxidoreductases.

Although there are no known agricultural impact yet of the research findings, the foundations for this research have been set and proven. Thus, continuation project should address our goals in detail and facilitate a sophisticated lab-on-chip technology, based on dual-detection-modes that would lead to a new generation of real-time water monitoring system. This system can help assuring water bodies’ safety and security and may considerably contribute to technological realization of other complex biosensing applications.

Publications for Project IS-5060-18

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Appendix:

Unpublished data:

Optical biosensing platform fabrication: Porous Si (PSi) thin-films were prepared by electrochemical anodization of highly doped p-type Si at a constant density of 83 mA cm^{-2} for 150 sec, as previously described.¹ Additionally, microcavity have been fabricated providing sharp spectral features for improved spectral sensitivity.²⁻⁴ The latter was etched by modulating high (H) and low (L) current densities according to the following sequence: $(\text{HL})_8\text{H}_4(\text{LH})_8$, where H is 60 mA cm^{-2} for 5 sec, L is 30 mA cm^{-2} for 10 sec and 5 sec intervals between each layer were used during the process to recover hydrofluoric acid concentration at dissolution edge. Subsequently, the resulting nanostructures were thermally oxidized (PSiO_2) at 600 and 800°C for microcavity and monolayer, respectively, to yield aqueously stable oxidized platform.⁵ Figure 1 depicts representative high resolution scanning electron microscopy (HRSEM) images of the resulting scaffolds. The porous monolayer layer is $5.0 \mu\text{m}$ thick with cylindrical shape pores characterized by 15-30 nm diameter, while the microcavity is slightly wider, pore diameter of 30-60 nm and $5.4 \mu\text{m}$ thickness. Figure 2 depicts characteristic reflectivity spectrum of the produced optical nanostructures. Briefly, the monolayer under perpendicular white light illumination consists of Fabry-Pérot fringe interference pattern ($m\lambda=2nL$), where m is an integer, n is the average refractive index, L is the thickness of the film and λ is the wavelength of the incident light (Figure 2a). Effective optical thickness (EOT) refers to the nL term in the Fabry-Pérot relationship.⁶⁻⁷ The fast Fourier transform (FFT) of this spectrum will display a single peak where the EOT of the porous layer can be extracted by the wavelength location of the peak, while the amplitude of this peak is related to the index contrast of each interface and light scattering at the porous layer surface.⁸ Similarly, the microcavity spectrum is monitored based on the characteristic peak dip $\sim 689 \text{ nm}$, without any additional analysis or mathematical manipulation (Figure 2b).³

Figure 1. HRSEM images of the characteristic layers: (a) top and (b) cross section of the produced monolayer; (c) top and (d) cross section of the microcavity structure.

Figure 2. The resulting reflectivity spectrum of a typical PSiO₂ (a) monolayer and (b) microcavity nanostructures.

The thermally oxidized nanostructures were biofunctionalized by standard silane chemistry.⁴⁻⁶ Briefly, the oxidized nanostructures were amino-modified by (3-aminopropyl) triethoxysilane (APTES) in alkaline conditions to activate the surface for glutaraldehyde (GA) grafting, in accordance with previous report.⁹ The homobifunctional cross-linker is acknowledged for its high reactivity with primary amines (both on APTES-modified surface and on the exterior of the specific bioreceptors). Next, specific biorecognition elements were immobilized onto the porous support by reacting prime amine with the second reactive group of GA. In case of copper sensing, branched polyethylenimine (PEI) with 25kDa was sequentially immobilized by applying two PEI layers on the solid support by homobifunctional cross-linking.¹⁰ The different synthetic steps were confirmed by reflective interferometric Fourier transform spectroscopy (RIFTS) that demonstrate EOT changes upon PEI functionalization cycles (Figure 3). Indeed, each modification modulates a positive change both in peak position and in peak intensity. We also used attenuated total reflectance Fourier

transform infrared (ATR-FTIR) spectroscopy in order to confirm the modifications. Table 1 summarizes peak areas of the different surface modifications both for protonated amines and for siloxane linkage. The amine modification ratio with respect to siloxane linkage following each functionalization step is increased. These results further verify RIFTS analysis that the PEI modification occurred.

Figure 3. Optical changes upon biofunctionalization of PSiO₂ with two sequential PEI layers.

Table 1. ATR-FTIR analysis of PEI modifications steps onto PSiO₂ surface.

Peak Area	ν (NH ₂) ^a	ν (Si-O-Si) ^b	Ratio (%)
PSi	0.05	101.5	0.05
APTES	0.17	60.9	0.28
PEI 1	0.59	64.9	0.91
PEI 2	0.70	55.5	1.26

^a 1520 – 1700 cm⁻¹

^b 860 -1280 cm⁻¹

The ratios of the areas under the amines groups peaks were normalized by the areas under the siloxane stretching mode peaks for each modification step.

Lead ions were identified through specific DNAzyme (5'-NH₂-T12-CATCTCTTCTCCGAGCCGGTCGAAATAGTGAGTCAGACATA-3') catalytic cleavage of single stranded DNA substrate (5'-NH₂-T12-TATGTCTGACTCACTAT-rA-GGAAGAGATG-3') modified with horseradish peroxidase (HRP) on the 5' (external side).¹¹ Lead ions induce hydrolytic cleavage at the scissile rA, thus dissociating the entire strand from the catalytic strand including the HRP. Figure 4a shows EOT changes following the bioconjugation of DNAzyme and its substrate with respect to GA modified PSiO₂ monolayer. The EOT values are increased with the attained modifications, presenting the construction of the biosensing platform. Figure 4b depicts fluorescence microscopy studies of fluorophore (fluorescein isothiocyanate, FITC) modified single stranded DNA substrate and subjected to different lead concentrations. Fluorescence intensity was quantified by CCD spectrometer (*Flame* series, Ocean Optics) coupled to a Nikon LV150N upright microscope. Indeed, the fluorescence signals react proportionally to lead ions excessive content, in which higher metal concentrations produces lower intensity values due to higher catalytic activity of the DNAzyme. The decrease is attributed to hydrolysis of the tagged substrate and its release from the surface. Complementary control experiments were carried out to ensure that the observed fluorescence intensity decrease was actually due to selective cleavage and subsequent release of the

substrate in the presence of lead rather fluorophore quenching. Moreover, specificity was also evaluated in mixture of other metals, i.e., mercury, cadmium, copper and zinc (data not shown).

Figure 4. (a) EOT changes upon biofunctionalization of P SiO_2 monolayer with DNAzyme followed by its substrate hybridization (100 and 50 μM , respectively); (b) Relative fluorescence intensity values obtained following admission of lead pollutants onto the DNAzyme and FITC modified substrate surface. Data are reported as mean $\pm$ S.D. ($n \geq 3$).

The selectivity of the platforms for multi-analytes was monitored through optical experiments using RIFTS. Changes in the reflectivity spectrum are monitored in real-time by acquisition of the reflectivity spectra of the porous thin-film upon target capturing. Wavelength shift or amplitude intensity were utilized for each sensing procedure. It is expected that the occurred interaction will modulate a noticeable complex formation (synthetic residue with copper ions) or enzymatic reaction products infiltration within the pores. This immediately translates into a shift in the reflectivity spectrum and correlates to wavelength or intensity changes. We have studied this concept upon introduction of different water samples spiked with metallic pollutants at various concentrations. Figure 5 depicts the optical response of PEI vs. different copper concentrations exposure. Increased copper concentration causes a profound growth in the relative intensity, as can be seen for 2000 ppb copper solution, an increase of 1.035 in the relative response is observed within ~ 7 min from the introduction of test solution. This behavior is ascribed to the primary amine groups of the PEI branched nanostructure interactions with the copper ions, as previously described by Kaur et al.¹⁰ Figure 5b presents the dynamic linear range of platform between 200 to 2000 ppb, while the calculated limit of detection is 54 ppb. To further confirm the feasibility of the developed assay in real water conditions and to evaluate the cumulative effect of abundant ions, we have performed sensing experiments of different water samples spiked with known copper concentrations. Table 2

summarized the overall performance of the PEI platform in tap, ground, and surface water samples. It should be noted that all samples showed insignificant copper content, below 1 ppb obtained by ICP-MS. The linear regression was used for calculating copper concentrations within all water samples. Overall, the recovery rates of the system (85 to 117%) reveal high selectivity toward copper ions and adaptability for complex media analysis (see Table 2).

Figure 5. PEI modified P SiO_2 optical response vs. different copper concentrations. (a) Relative intensity response towards copper concentrations (0–2000 ppb). The biosensor is fixed in a custom-made flow cell and the reflectivity spectra are recorded every 30 s; (b) The corresponding calibration curve of the relative intensity kinetics within the linear optical response. Data are reported as mean $\pm$ S.D. ($n \geq 3$).

Table 2. Copper sensing experiments in real water samples.

Sample	Spiked (ppb)	Detected (ppb)	Recovery (%)
Tap	300	275 $\pm$ 25	92
	500	563 $\pm$ 67	113
	1000	917 $\pm$ 68	92
Surface	300	280 $\pm$ 27	93
	500	462 $\pm$ 13	92
	1000	1165 $\pm$ 42	117
Ground	300	278 $\pm$ 35	93
	500	425 $\pm$ 52	85
	1000	1041 $\pm$ 24	105

Linear regression was used for calculating copper concentrations within all water samples. Data are reported as mean $\pm$ S.D. ($n \geq 3$).

Figure 6 shows the optical response of DNAzyme sensing concept on both P SiO_2 platforms amplified by the enzymatic activity of HRP.^{5, 12–13} The optical responses of monolayer and

microcavity (normalized EOT and peak shift, respectively) are profoundly decreased upon lead content augmentation. Both platforms present high sensitivity towards lead ions, while sensing concentrations as low as 10 ppb within 50 min of bioassay were obtained. Figure 6a depict dehybridization control experiment, in which both strands were detached, thus releasing the HRP residues. The resulting deactivates the optical response of the platform as insignificant normalized EOT values were attained ($<0.05\%$).

Figure 6. DNAzyme modified PSiO₂ optical response vs. different lead concentrations. The surfaces (a) Monolayer and (b) Microcavity are incubated with various lead ion solutions, followed by injection of (1) 0.8 mM 4-chloro-1-naphthol (enzymatic substrate) in HEPES buffer (pH 7.4); (2) The HRP residue on the substrate's 5' is activated by the addition on hydrogen peroxide to the cycled solution. The biosensor is fixed in a custom-made flow cell and the reflectivity spectra are recorded every 30 s.

Electrochemical Sensing Platform:

Cyclic voltammetric (CV) experiments were carried out extensively first to assess direct electron transfer between the microbial cells and the electrode and to evaluate the capability of two mediators, p-benzoquinone and the redox polymer NQ-LPEI to mediate the extracellular electron transfer process. *P. veronii* 2E response in the presence of 20 mM glucose was studied by CV at 1 mV s⁻¹ on carbon paper bioanodes. As shown in Figure 7, the voltammograms of the bare carbon paper electrodes (CPE, black line) and the autoclaved cells immobilized on CPE (red line) show no significant current response to glucose in the anodic region of the curve. Immobilized living *P. veronii* 2E cells generated a catalytic response directly associated with bacterial metabolism (blue line).

Figure 7. CVs for *Pseudomonas veronii* 2E on carbon paper electrodes (blue), and control experiments with autoclaved cells (red) or cell-free electrodes (black) in 20 mM MOPS buffer (pH 7) + 10 mM MgCl_2 . Substrate: 20 mM glucose. Scan rate 1 mV s^{-1} . RE: Saturated calomel electrode. CE: Pt.

While direct electron transfer between bacterial cells and the electrode was possible, only limited current values were achieved, motivating our interest in exploring different approaches of mediated electron transfer. Accordingly, *P. veronii* 2E cells were immobilized in a calcium alginate matrix to increase cell density on the electrode and 0.3 mg mL^{-1} of activated carbon was incorporated to the matrix to improve conductivity. p-BQ was added to the cell-alginate suspension as an exogenous electron transfer mediator, since its capability to diffuse through the cellular membrane of bacterial cells and act as a redox shuttle has been reported for several bacterial species. CV response for this type of electrode is reported in Figure 8. The appearance of a redox peak with a formal redox potential of $+0.015 \text{ V vs. SCE}$ confirmed that the mediator was successfully immobilized in the matrix (Figure 8 A line black). Furthermore, a biocatalytic current signal was observed for the bioanode system with *P. veronii* 2E p-BQ+AC carbon felt electrode (Figure 8 line blue). In order to explore a more sustainable mediating approach, the anode was modified with the NQ-LPEI redox polymer where the naphthoquinone redox moieties resembling redox carriers present in bacterial cells are confined on the polymer backbone. The polymer allowed enhancing EET also for *P. veronii* 2E, resulting in a bioelectrocatalytic current (Figure 8B, blue line, $-16 \pm 4 \mu\text{A cm}^{-2}$) greater than the one obtained for bioanodes with p-BQ + AC mediator system ($-9 \pm 5 \mu\text{A cm}^{-2}$).

Figure 8. CVs for *Pseudomonas veronii* 2E immobilized on carbon felt electrodes modified with calcium alginate capsules containing 0.7 mM p-BQ+AC (A) or NQ-polymer (B) (blue). Control experiments with autoclaved bacteria (red) or cell-free electrodes (black) in 20 mM MOPS buffer (pH 7) + 10 mM MgCl₂. Substrate: 20 mM glucose. Scan rate 1 mV s⁻¹, counter electrode Pt, reference electrode SCE.

Chromium (VI) effect and online monitoring

The effect of Cr(VI) on the biocatalytic response of *P. veronii* 2E was first explored by CV utilizing a Cr(VI) shock of 50 mg L⁻¹. As shown in Figure 9, the current signal was considerably suppressed in the presence of this contaminant in both mediated systems, opening for the possibility to utilize the developed system for Cr(VI) monitoring.

Figure 9. CVs for *Pseudomonas veronii* 2E with Cr(VI) addition in 20 mM MOPS buffer (pH 7) + 10 mM MgCl₂, 0 mg L⁻¹ Cr(VI) (Continuous line), 50 mg L⁻¹ Cr(VI) (Dash line). *P. veronii* 2E immobilized in calcium alginate with 0.7 mM p-BQ+AC (A) or NQ-polymer (B) (blue). Control experiments with autoclaved bacteria (red) or cell-free electrodes (black). Substrate: 20 mM glucose. Scan rate 1 mV s⁻¹, counter electrode Pt, reference electrode SCE.

Accordingly, in order to explore a possible quantitative application of Cr(VI) inhibitory effect, both mediated systems were exposed to different Cr(VI) concentrations. For p-BQ+AC calcium alginate immobilization, 0.2, 0.4 and 0.7 mM p-BQ were used in the assembly with no modification of AC content. First, it should be noted that a small change in current production is obtained when changing p-BQ concentration in the absence of chromium, resulting in $-7.2 \pm 0.6 \mu\text{A cm}^{-2}$, $-10 \pm 3 \mu\text{A cm}^{-2}$, and $-9 \pm 5 \mu\text{A cm}^{-2}$ for 0.2, 0.4, and 0.7 mM p-BQ, respectively at E +0.25 V. After the addition of different Cr(VI) concentrations, a considerable dispersion of the current response was observed. Interestingly, it can be noted that the best linear response was obtained for the cells immobilized with 0.7 mM p-BQ, while a more significant inhibition of the response was obtained for lower concentrations of the mediator. Such a trend could be explained considering that p-BQ can reduce small amounts of Cr(VI). Accordingly, the higher concentration of p-BQ could reduce the amount of Cr(VI) reaching the bacterial cells, allowing to obtain a linear calibration curve in a range of 4 - 18.5 mg L⁻¹ Cr(VI) ($R^2 = 0.94$).

The use of NQ-LPEI redox polymer also resulted in a linear current response to Cr(VI) concentration, showing higher sensitivity ($0.31 \pm 0.02 \mu\text{A cm}^{-2} \text{ mgCr(VI)}^{-1} \text{ L}$, $R^2 = 0.98$) compared to the bioanode with p-BQ as a redox mediator ($0.22 \pm 0.04 \mu\text{A cm}^{-2} \text{ mgCr(VI)}^{-1} \text{ L}$, $R^2 = 0.94$), and allowing for a linear range between 4 and 18.5 mg L⁻¹ of Cr(VI). While the linear range obtained for the two bioanode configurations are similar, the use of the NQ-LPEI redox polymer is preferable over the use of entrapped p-BQ for two reasons: i) for the former the redox moieties are immobilized on the polymer backbone avoiding their release in solution; ii) the low redox potential of the NQ-LPEI allows harvesting the electrons obtained from bacterial metabolism with a lower over potential.

Analyzing the performance of the biosensor in view of current legislation, it is important to note that even if the biosensor employing NQ-LPEI ensures a lower limit of detection (LOD) (2.4 mg L⁻¹) and limit of quantification (LOQ) (8.1 mg L⁻¹) compared to the configuration employing p-BQ (0.7mM)+AC (LOD: 6.4 mg L⁻¹, LOQ: 21.2 mg L⁻¹), such performance does not allow reaching the required limits for chromium release in industrial wastewaters. However, the poor detection limits are due to the system being in transport limited mode, where you are limited by transport of the analyte across the cellular membrane. Therefore, the oxidoreductase must be isolated and purified from the Pseudomonas bacteria and then immobilized within the NQ-LPEI/activated carbon composite. Due to the shortened one year time period, we will be unable to accomplish this next step,

but current densities for enzyme systems in NQ-LPEI are on the order of mA/cm^2 instead of $\mu\text{A}/\text{cm}^2$, so we expect impressive improvements in sensitivity and detection limits.

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