Development of a Biosensor for Detection of Phosphate Species in Uranium Contaminated Ground Water and Wastewater Sediments

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ABSTRACT

Detection of phosphate ions in the groundwater at the Hanford site is essential for monitoring the stability of uranium in the field because it plays an important role in binding and precipitation. Since environmental factors can influence the stabilization of uranium via polyphosphate binding, the presence of phosphate ions in groundwater, soil and lake sediments must be determined. The stabilization of uranium via polyphosphate injection has been investigated by Wellman et al. It has been demonstrated that the reduced concentration of phosphate can reduce the stabilization of uranium. On the other hand, high concentration of phosphate in water causes eutrification. In addition, when polyphosphate is added to the uranium contaminated area, stabilization of uranium as apatite and sequestered as autunite has been reported by Wellman. Therefore, the investigation and quantification of the phosphate species is essential for understanding the remediation and leaching processes involved with uranium. Despite the past research and previously developed phosphate detection devices, an improved PO_4^{3-} sensor that is field deployable is still needed. Here we report on the development of sensors capable of detecting the concentration of phosphate species in uranium contaminated areas at the Hanford site. The method of detection is based on the immobilization of superior biocatalyst(s) on superconductive metal alloy(s) and micro-cantilever(s). Several phosphate related proteins were studied as biocatalysts for developing the enzyme sensors for the phosphate detection and quantification. The success of the experiments, however, is limited due to the activity and durability of the utilized enzymes, the conductivity of the materials used, and the sensitivity of the sensors. Even though the project is still ongoing, we have obtained preliminary data.

INTRODUCTION

Uranium contamination at the Hanford site is a major environmental predicament caused by both human and natural factors which are two important uranium sources. In order to prevent the leaching of uranium to the groundwater, it needs to be stabilized in the subsurface. Contamination of this radionuclide in groundwater occurs at several sites in the United States (1**).** At many of these sites, the concentration of uranium exceeds the standards (30μg/L) set by the U.S. Environmental Protection Agency (2). Currently, DOE scientists are focusing on polyphosphate attachment techniques for immobilizing uranium in the soil. The stabilization is based on the direct binding of the polyphosphate compounds to uranium via electrostatic interactions with a highly positive charged element. Uranium is usually available in the environment either as a reduced form, U^{4+}, or as the oxidized form, U^{6+}. Uranium +6 has a tendency to bind polyphosphate and oxidize to the +4 state (3, 4, and 5). When polyphosphate is

added to the uranium contaminated area, stabilization of the uranium as apatite and sequestered as autunite has been reported by Wellman et al. This research revealed that reduced concentrations of phosphate can reduce the uranium stabilization (6).

Phosphate is not only essential for uranium stabilization, it is also one of the vital nutrients for all living organisms (7). It can be found in nature (e.g., water sediments) (8), and it often exists in an inorganic form. The amount of phosphate in the environment strongly influences the operations of living organisms. For instance, a high concentration of phosphate in water causes eutrification which is a subject of utmost concern, and has been recognized by the European Union through legislation that stipulates 0.1 mg/L [PO_4^{3-}]. The excess amount of phosphate is an indicator for possible problematic algal growth in rivers (9). In contrast, low phosphate concentration causes death of vegetation since plants utilize the inorganic phosphate for photosynthesis, respiration, and the regulation of enzymes (8). Since phosphate plays an important role in the soil and groundwater sediments both for uranium and living organisms at Hanford as well as to ensure consistent water quality, the phosphate concentration should be quantified and monitored in order to deploy the right quantity for remediation. Throughout the years, these factors encouraged the scientists to investigate methods for phosphate detection (10, 11). Up to now, several sensor technologies for monitoring phosphate species have been implemented. Among these, a microfluidic system with a 0.7 ppb detection limit, an autonomus microfluidic system with a 3µM detection limit, a biosensor based on the pyruvate oxidase modified conducting polymer for phosphate ion determination with a 3µM detection limit, and a thick-film phosphate biosensor based hydrogel immobilized pyruvate oxidase with a 5 µM detection limit are a few that have drawn particular attention (12, 13, 14 and 15). Table 1 summarizes the detection limits of phosphate from various techniques.

Table 1. Phosphate Concentration Detection Limits with Different Methods (10)

Method	Detection Limit (µM)
FIA-P	100
ISE Molybdate complex	0.06
Amperometric Molybdate complex	0.3
Amperometric POD/O2	1
Amperometric POD/H2O2	3.6
Amperometric NP, XOD, AP	0.01
Amperometric MP, MR, GOX, AP	0.01
Ion chromatography	0.1
Capillary electrophoresis	0.1
Luminescent plate Europium–tetracycline	3
Fluorescent probe NP, XOD, HRP	0.05
Fluorescent PVC matrix Al-morin ionophore	0.2

Although these sensors have successfully detected the phosphate, they have peculiar drawbacks. For instance, conventional colorimetric and potentiometric techniques have disadvantages in sensing. The prominent downside is that the systems are too complicated for frequent use or low selectivity (10,16 and 17). Enzyme-immobilized biosensors are the most applicable ones for the

phosphate detection and quantification because of their substrate specificity and lower detection limits. In terms of frequent use, the enzyme utilized in the biosensor has to be replaced due to the descending or limited time of enzyme activity. Although the bio- catalytic sensors have these types of weaknesses, they offer a quick response and provide very rapid results. The first enzymatic phosphate biosensor was developed employing double enzymes by Guilbalut et al. (11, 18). This group utilized both alkaline phosphatase and glucose oxidase for their biosensor. However, alkaline phosphatase becomes active on the functionalities of phosphate ester and the derivatives of alcohol by opening the bond between them which lowers the enzyme specificity to the substrates. Then again, the sensing system could not be used in the aquatic environment for the lower concentrations of phosphate detection (10). Later, Wollenberger et al. attempted to lower the detection limit of the phosphate concentration by employing three different enzymes simultaneously in their research (11, 19). Although this group was able to decrease the detection limit by 10-fold as compared to Guilbalut's design, the response time of the multiple enzymatic sensors was about three minutes for a single measurement as opposed to seconds with biosensors. Furthermore, the function of its selectivity negatively affected the long term stability of the sensor (14, 20). The single enzymatic method using pyruvate oxidase, first introduced by Mizutani et al. in 1983, is the most efficient and highly selective to phosphate (21). Moreover, the immobilization of a single enzyme is simpler than the immobilization of double or triple enzymes to the surface and is more effective. It is reported that the double and the triple enzyme-immobilized sensor technology has some disadvantages because the enzyme shelf lives are not harmonious. Also, the conventional pyruvate oxidase method suffered from storage stability, low reproducibility, and selectivity due to the poor design (21, 22). The current phosphate detection method used at Hanford is sampling and analysis at the laboratory which has many disadvantages (i.e., potential cross contamination, longer turnaround time and high cost). For these reasons, we are developing a new biosensor to detect phosphate in groundwater by employing advanced materials and nano-materials to overcome these matters.

MATERIALS AND METHODS

Materials

Pyruvate oxidase (POX) (EC. 1.2.3.3, 100 units mg−l) from aerococcus species, 1-ethyl-3 (3-(dimethylamino) - propyl), 50mM Tris Buffer pH 7.5, 50mM HEPES Buffer pH 7.5, DMSO (Dimethyl sulfoxide), sodium dihydrogenphosphate, potassium dihydrogen phosphate, sodium pyruvate, thiamine pyrophosphate chloride (TPP), flavin adenine dinucleotide (FAD), and *N*-2-hydroxyethylpiperazine were purchased from Sigma Aldrich Co. (USA) and used as received. All aqueous solutions were prepared in doubly distilled water, which was obtained from a Milli-Q water purifying system (18M_cm).

Apparatus

Pyruvate oxidase was immobilized on gold-itself, gold-coated silicone polymers and superconductive alloys. The following electrodes were employed for the amperometric measurements; glassy carbon electrode (GCE) as a working electrode, Ag/AgCl (in saturated KCl) as a reference electrode and platinum wire as a counter electrode. Cyclic voltammetry (CV) and amperometric measurements were performed by using the Potentiostat/Galvanostat Solartron

model (SI-1287 and SI-1260) electrochemical measuring devices. Micro-electrochemical measurement cells (Figure 1) were constructed from plexiglas materials.

Fig. 1. The custom-made micro electrochemical measuring cell.

Preparation of Enzyme-Linked Advanced Materials

For the development of the enzyme-linked biosensor via advanced materials, three different types of materials were employed and their efficiency, based on the electron transfer rate, were investigated and compared to each other: gold surface itself, a gold-coated silicone based polymer and superconductive metal alloys with Multi Wall Carbon Nano Tube (MWCNT). Au was deposited on the silicon polymers and the metal alloys by evaporating the gold in a vacuum chamber (thermionic vacuum system). The single enzyme was immobilized on the gold or gold coated materials through an organo-chemical linker. On the other hand, a powder metallurgical technique was used to prepare MWCNT based on the metal matrix composite (MMC) compacts. These compacts were believed to possess high electrical conductivity and the addition of MWCNTs will impart binding to the composite. The electrical resistivity of the sintered compacts was measured using a four probe HP 4263A LCR meter. The four probes were attached equi-distance on the surface of the compact using indium to minimize surface contact resistance. The resistivity was calculated using the formula: $r = (p \, .t / \ln 2)V / I$ for $s>>t$ where r is the resistivity, R=V/I is the resistance, s is the distance between the probes and t is the thickness of the sample. The electrical conductivity was determined as the reciprocal of the electrical resistivity. A schematic representation of the four-point probe is shown in Figure 2.

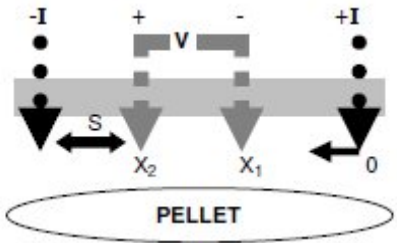

Fig. 2. Schematic representation of the four-point probe (23).

Electrochemical Measurements

The electrochemical measurements were recorded in the custom-made electrochemical microcell (Figure 1). In order to ensure that there was no interference available in the solution, the CV graph shown in Figure 2 was recorded by using a freshly prepared 50 mM Tris buffer, pH 7.4. The steady-state current of the buffer was monitored and pH and temperature were optimized by continuous stirring. For the phosphate quantification via enzyme-linked sensory systems, each amperometric measurement was recorded by injecting different concentrations of phosphate solutions onto the Tris buffer in the cell. To prevent cross-contamination, the used solutions (Tris buffer+phosphate solution) were discarded after each amperometric titration.

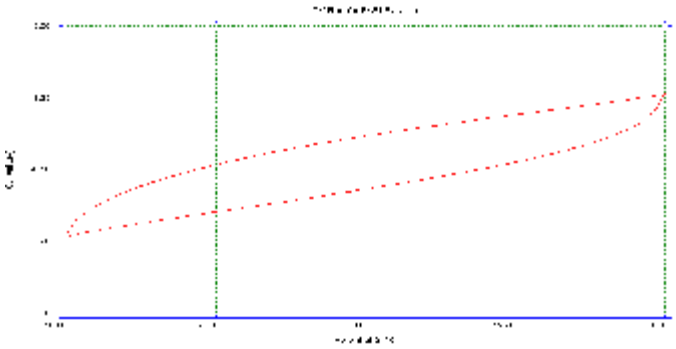

Fig. 3. CV of background buffer solution.

RESULTS AND DISCUSSIONS

Each experiment was done in the same order. For the electrochemical measurements, both cyclic voltammetry and Amperometry, the conventional three electrode system was used; glassy carbon electrode, Ag/AgCl, and platinum wire as working, reference and counter electrode, respectively. In this research, the pyruvate oxidase was utilized as a biocatalyst (22).

The reaction catalyzed by this enzyme is:

$$\text{Pyruvate} + PO_4^{3-} + O_2 \xrightarrow{\text{POX}} \text{Acetyl P} + H_2O_2 + CO_2$$

From the reaction, H_2O_2 is released as a byproduct and it is detected between the working electrode and the counter electrode. Before and after each usage, the enzyme immobilized materials were washed 3 times with Tris buffer to eliminate any potential for contamination. All the measurements were done at ambient temperature.

Investigation of Silicon Based Gold-Coated Enzyme Carrier

Because of the substrate specificity of the enzymes, we immobilized pyruvate oxidase onto the gold-coated advanced materials. In order to obtain preliminary data and test the capacity of the advanced materials, a single enzyme was attached to the gold-coated silicon based material. FAD, TPP and Mg^{2} added 1 ml Tris buffer was placed in the cell shown in Figure 1. Then, the Au-coated silicon was placed in the cell. The phosphate compound in the form of $Na_2HPO_4^{3-}$ was injected into the buffer solution in different concentrations.

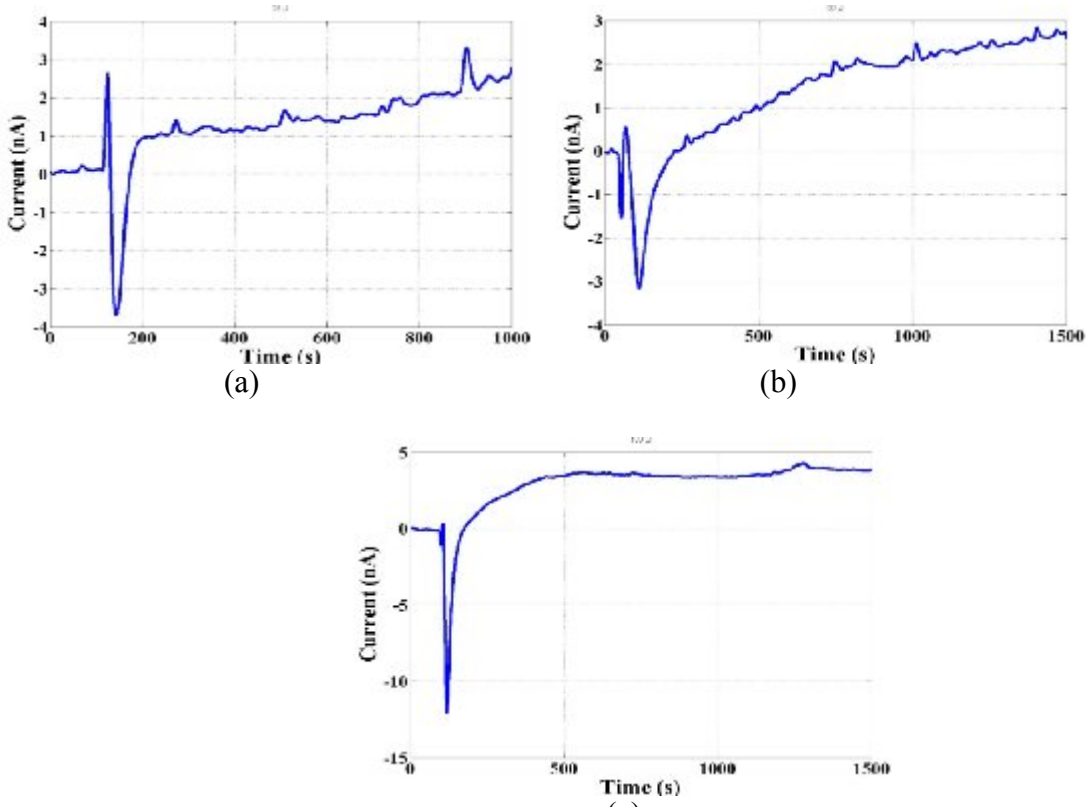

Fig. 4. Amperometric graphs obtained in varying amounts of phosphate addition.

Figure 4 shows a typical amperometric current-time plot with different amounts of phosphate added to the Tris buffer. The applied potential was +0.4V and the plot obtained is in current versus time; the settings were kept the same for each experiment. The current increase in Figure 4a was noticed as well as in Figure 4b and Figure 4c. However, the increment slope was different due to the varying of the PO_4^{3-} concentration. Figure 5 clearly indicates the slope distinction between different concentrations. A calibration curve was established (Figure 6) by using obtained slopes. As preliminary data, the phosphate range was detected between 10 mM to 100mM.

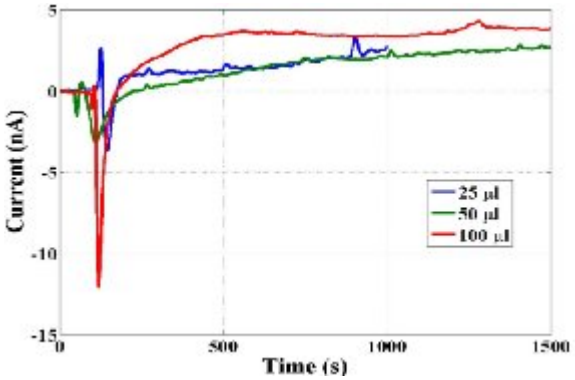

Fig. 5. Current vs time with varying phosphate concentrations.

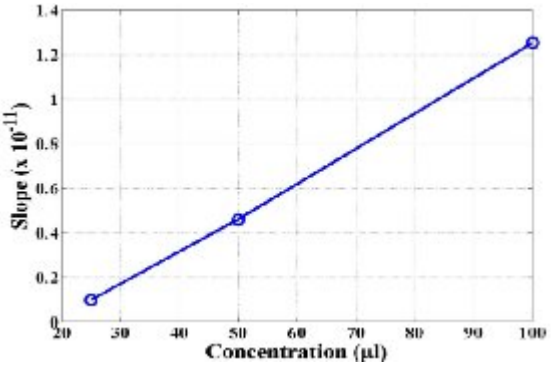

Fig. 6. Calibration curve.

The preliminary data of the newly developed biosensor promises more accurate and sensitive PO_4^{3-} detection for the Hanford site. The construction of the biosensor is underway. Therefore, additional research will need to be performed in order to disclose the effectiveness and the capacity in terms of insitu applications. Wellman et al. reported that the concentration of phosphate at the Hanford site is in the range of 10^{5-} mM to 52.6 mM (23). The development of biosensor research for phosphate detection in the environment will continue until these levels are achieved. Thus far, we have measured between 10-100 mM phosphate concentrations in the Tris buffer via employing newly developed sensors. Since the research is still underway, we are targeting to lower the phosphate detection limits as well as to test their field application capacity. The shelf life of the enzyme sensors are one of the most important aspects; therefore, we will investigate the maximum durability of the sensors. According to the literature, the stability of the multi enzyme-linked sensors depends upon the life-span of the enzyme. In order to increase the durability of the sensor, the single enzyme technology was chosen to develop the phosphate sensing system.

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