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Removal of binary Cr(VI) and Cd(II) from the catholyte of MFCs and determining their fate in EAB using fluorescence probes☆

Liping Huang [a,*], Peng Zhou [b], Xie Quan [a], Bruce E. Logan [c]

[a] Key Laboratory of Industrial Ecology and Environmental Engineering, Ministry of Education (MOE), School of Environmental Science and Technology, Dalian University of Technology, Dalian 116024, China
[b] College of Chemistry, Dalian University of Technology, Dalian 116024, China
[c] Department of Civil and Environmental Engineering, The Pennsylvania State University, University Park, PA 16802, United States

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ABSTRACT

Electrochemically active bacteria (EAB) on the cathodes of microbial fuel cells (MFCs) can remove metals from the catholyte, but the fate of metals in the cells has not been examined in the presence of multiple metals. To study the relative uptake and fate of Cr(VI) and Cd(II) in cells, fluorescence probes were used to determine the amount and location of these metals in four different EAB on the biocathodes of MFCs. When both metals were present, less Cr(VI) was removed but Cd(II) uptake was not appreciably affected. As a consequence, the imaging of Cr(III) ions was lower than that using individual fluorescence probes for single Cr(III) ions in each EAB, compared to negligible changes in images for Cd(II) ions in the presence of either both Cr(VI) and Cd(II) or Cd(II) alone. The concentration of Cr(III) ions in the cells consistently increased over time, while that of Cd(II) ions decreased following an initial increase. Cr or Cd uptake could not be detected using a scanning electron microscope coupled with an energy dispersive spectrometer, reflecting the high sensitivities of the fluorescence probes to these metals. More chromium was found in the cytoplasm while cadmium preferentially accumulated in the cell envelope. These results demonstrate that the fate of chromium and cadmium in EAB was different when both metals were present, compared to controls containing a single metal. These results provide direct and visible results on the fate of the metals in the EAB when these metals are co-present in the catholyte of MFCs.

1. Introduction

Bacteria are capable of removing metals to very low concentrations, and thus they have attracted great interest for applications in metals remediation [1,2]. Bacteria that grow on the cathodes of microbial fuel cells (MFCs), called electrochemically active bacteria (EAB), can accept electrons and reduce oxygen or a variety of different metals making it possible to remove metals while simultaneously generating electricity [3–8]. Metals removal by a biocathode is particularly useful for removing toxic Cr(VI) and Cd(II), which are usually both present in a variety of metal-processing wastewaters [1,2,9]. Most studies on these metals have examined only the removal of a single metal from solution despite the presence of both metals in actual wastewaters [6,10–14]. However, for practical bioremediation applications, it is desirable to simultaneously remove both metals from solution.

The fate of Cr(VI) and Cd(II) within the cell has not been well examined, particularly from the perspective of their simultaneous presence in a wastewater. Soluble Cr(VI) can be reduced to Cr(III)-complexes and precipitated $Cr(OH)_3$ via the transiently formed Cr(III) ions, but when Cd(II) is present it can form organic metal-complexes containing or inorganic precipitates containing both metals. These metals can be found on the bacterial outer membranes, or in the cytoplasm or periplasm. Previous studies have examined the uptake of these metals using techniques such as global kinetic analysis and titrimetry [15–17]. The presence of these metals in the cells has been investigated using many different techniques, including X-ray absorption near-edge structure (XANES), extended X-ray absorption fine structure (EXAFS), inductively coupled plasma mass spectroscopy (ICP-MS), low frequency electron spin resonance (ESR) spectrometry, X-ray photoelectron spectroscopy (XPS), raman spectroscopy, scanning electron microscope equipped with energy dispersive spectroscopy (SEM-EDS), and transmission electron microscopy equipped with energy dispersive spectroscopy (TEM-EDS) [18,19]. However, these techniques do not quantify the amount of metal take up, or the locations of transiently formed Cr(III) ions and the permeated Cd(II) ions inside of bacteria. Thus, different techniques are needed to assess the concentration and location of Cr(III) and Cd(II) ions on or in the cells.

Fluorescent probes for metals have recently been shown to be useful as a highly sensitive, rapid, and nondestructive way to visually and quantitatively identify metals in cells [20,21]. These probes have

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* Corresponding author.
E-mail address: lipinghuang@dlut.edu.cn (L. Huang).

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individually identified Cr(III) [22] or Cd(II) [23] in bacteria, but they have not been simultaneously used to track the location of these metals. In most practical situations both metals are often present, and it is not known how the presence of both these metals might impact the fate of the metals in the cells. Thus, simultaneous imaging sensing of Cr (III) and Cd(II) ions in EAB could reveal whether different amounts of the metals were taken up in the presence of both metals, and show whether the preferential location of these metals might change compared to tests with individual metals. An understanding of the fate of these metals could lead to improvements in the removal effectiveness of bioremediation using biocathodes and other electrochemical processes for in-situ remediation.

In order to quantify the uptake and fate of both Cr(VI) and Cd(II) metals when both metals were present, pure culture MFC experiments were conducted using four known EAB that were isolated from Cr(VI) reducing biocathodes, *Stenotrophomonas* sp. YS1, *Stenotrophomonas maltophilia* YS2, *Serratia marcescens* YS3, and *Achromobacter xylosoxidans* YS8 [22,24]. As controls, the distribution of subcellular chromium in the presence of only Cr(VI) in catholyte and subcellular cadmium in the presence of single Cd(II) in catholyte was also mapped. Using pure culture strains allow us to directly understand the EAB at the electrode surface for Cr(VI) and Cd(II) removal whereas comparing the EAB behaviors is beneficial for understanding the distribution of various valence states of chromium and cadmium in the EAB. A naphthalimide-rhodamine based Cr(III) probe [25] and a quinoline-based Cd(II) probe [26] were simultaneously used to evaluate the uptake and fate of the two metals added alone or together to the cathodes of MFCs with current generation, and compared to open circuit controls or abiotic cathodes. Cr(III) and Cd(II) ions were then identified in the protoplasm and in the cytoplasm of the cells.

2. Materials and methods

2.1. Inoculation and MFC operation

Pure cultures of the four EAB (YS1, YS2, YS3, and YS8) were incubated in an anaerobic medium (AM, pH = 5.8) containing: NH_4Cl (5794 μM), KCl (1743 μM), $MgCl_2 \cdot 6H_2O$ (885 μM), $CaCl_2 \cdot 2H_2O$ (612 μM), KH_2PO_4 (11.0 mM), $K_2HPO_4 \cdot 3H_2O$ (12.7 mM), and 1 mL of trace elements [22].

Duplicate two-chamber MFCs were used in all experiments as previously described [22]. Briefly, porous graphite felt (each piece: 1.0 × 1.0 × 0.5 cm, with a total of 8 pieces; Sanye Co., Beijing, China) was inserted with carbon rod collectors and used for the cathodes and anodes [22,27,28]. The anode (26 mL) and cathode (13 mL) chambers were separated by a cation exchange membrane (CEM) (CMI-7000 Membranes International, Glen Rock, NJ) with a projected surface area of 7.1 cm^2 [29]. The anodes were inoculated using the effluent of MFCs well acclimated to acetate (12.2 mM) [30,31]. The electrolyte in both chambers was AM, except for the catholyte where acetate was replaced by $NaHCO_3$ (119 μM). Solution conductivities were adjusted to be 5.8 mS/cm using 0.1 M KCl [32,33]. Both the anolyte and catholyte were bubbled with ultra-pure N_2 for 10 min, and then sealed in order to maintain anaerobic condition during the experiments. The MFCs were operated at a fixed external resistance of 510 Ω, and a reference electrode (Ag/AgCl, 195 mV vs. SHE) was placed in the cathode chamber to measure the cathode potential. All potentials were reported vs SHE. All reactors were wrapped with aluminum foil to exclude light.

Cathodes were inoculated with one of the four different isolates (6 mL containing 10^8 CFU/mL), and each cycle of MFC operation was set at 5 h. Concentrations of chromium and cadmium in actual wastewaters can vary over a large range of 0.008–5190 mM [2,30,31]. For metal reduction experiments here, Cr(VI) (385 μM) and Cd(II) (179 μM) were added to AM at concentrations within this range, consistent with previous studies on the activities of these microorganisms [14,22,23]. Three replicate experiments were performed with both metals using duplicate reactors with current generation. Three types of reactors (in duplicate) were used as controls: one was operated without the inoculum (abiotic control); the second was inoculated but operated under open circuit conditions (OCC) to examine removal in the absence of current generation; and the third was operated in the presence of only a single metal to contrast changes with two metals with that of Cr(VI) or Cd(II) alone in the system.

2.2. Preparation of Cr(III) and Cd(II) fluorescent probes

The fluorescent probes were synthesized for Cr(III) ions as described by Mao et al. [25], and for Cd(II) ions by Xu et al. [26] (Fig. S1). The chemical properties of the probes were confirmed by high resolution mass spectrum (HRMS) data, and 1H and 13C nuclear magnetic resonance spectra (NMR) as previously described [22,23].

2.3. Assessment of toxicity and selectivity of fluorescence probes

Prior to imaging studies, cell viability in the absence and presence of both fluorescence probes (each at concentrations of 5, 10, 20, 30 μM) was assessed using 96-well plates based on growth in AM. Briefly, EAB grown in AM for 24 h were treated with fluorescent probes and incubated for another 24 h at 30 °C. A FD FACSCanto flow cytometer (Biosciences) was used to assess the viability [22]. Cell survival in the presence of the probes were normalized to the untreated control cells. Experiments were performed at least 3 times.

In order to assess selectivity of the probes, bacteria were first incubated in the absence of Cr(VI) and Cd(II). Cell periplasm and cytoplasm were then prepared through ultrasonic cell disruption, osmotic shock and centrifugation as described in SI [22,23,28] before exposed to the probes and metal ions. The selectivity of Cr(III) and Cd(II) probes toward Cr(III) and Cd(II) ions in the periplasm and cytoplasm samples was examined using either Cr(III) or Cd(II) ions, and then both Cd(II) or Cr(III) ions. Finally, in the presence of both fluorescence probes and Cr(III) and Cd(II) ions, fluorescence intensities were examined before and after adding other individual metal cations [Zn(II), Fe(III), Co(II), Mn(II), Ni(II), Ca(II), K(I), Na(I)]. Standard curves for the responses of Cr(III) or Cd(II) probes to Cr(III) or Cd(II) ions in either the cytoplasm or periplasm of the four bacteria, and detection limits of these probes, were established based on the description of cytoplasm and periplasm preparation in SI. Relative errors ranged from 9 to 17%.

2.4. Imaging of Cr(III) and Cd(II) ions in live cells

Images for Cr(III) and Cd(II) ions in live cells were examined using confocal laser scanning microscopy (CLSM) (Olympus FV 1000, Japan). Briefly, cells were incubated with both probes (10 μM each) at 30 °C for 20 min, rinsed three times by centrifugation (6000 ×g), and re-suspended in saline water (1 mL). A sample (100 μL) was placed on a clean coverslip and covered with agar (3 mL) at 40 °C for 20 min. Cells were then observed using CLSM under 400 × oil-immersion objective lens (Green channel (520–620 nm), λ_{ex} = 488 nm; red channel (420–480 nm), λ_{ex} = 405 nm). Fluorescence microscopy images were normalized to the brightest sample of the entire set using ImageJ software (NIH, Bethesda, MD). Image analysis and fluorescence quantification was conducted according to the procedure of Kellenberger et al. [34] (see details in the SI). The statistical significance ($p \le 0.05$) of differences between samples was assessed using a statistical package (*t*-test, SPSS v.19.0). Two controls were performed: cell suspensions in the absence of fluorescence probes, and addition of the probes in the absence of cells.

2.5. Measurements and analyses

Average current was calculated based on the sum of current for each operation interval divided by entire operation time (5 h). Polarization