Electricity Generation by *Geobacter sulfurreducens* Attached to Gold Electrodes

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The versatility of gold for electrode manufacture suggests that it could be an ideal material for some microbial fuel cell applications. However, previous studies have suggested that microorganisms that readily transfer electrons to graphite do not transfer electrons to gold. Investigations with *Geobacter sulfurreducens* demonstrated that it could grow on gold anodes producing current nearly as effectively as with graphite anodes. Current production was associated with the development of *G. sulfurreducens* biofilms up to 40 μm thick. No current was produced if *pilA*, the gene for the structural protein of the conductive pili of *G. sulfurreducens*, was deleted. The finding that gold is a suitable anode material for microbial fuel cells offers expanded possibilities for the construction of microbial fuel cells and the electrochemical analysis of microbe−electrode interactions.

Introduction

Microorganisms that produce electricity by oxidizing organic compounds with electron transfer to electrodes may be useful agents for current generation from waste organic matter and renewable biomass, as well as for sensors.[1−4] Graphite has typically been the material of choice for the construction of anodes of microbial fuel cells. However, other conductive materials may be preferable, either because they enhance electron transfer between the microorganisms and the anode material or because they are better adapted to specific applications. For example, incorporation of manganese, iron, quinones, or neutral red in graphite electrodes increased the output of microbial fuel cells.[5,6]

Gold is a potentially attractive anode material for some microbial fuel cell applications because it is highly conductive and because gold provides a high degree of versatility for electrode manufacture. However, previous studies with *Shewanella putrefaciens* suggested that bare gold is a poor electrode material for the anode of microbial fuel cells. Current production with gold electrodes was low and increased 100-fold when the gold surface was coated with a surface-associated monolayer (SAM) of 11-mercapto-undecanoic acid,[7] even though the SAM would be expected to have insulating properties.[8] These results indicated that the gold surface was either toxic to the cells or otherwise poorly suited to interact with electron-transfer cell components. Redox-active proteins, such as cytochromes, may adsorb strongly to gold resulting in denaturation and loss of their electron-transfer capabilities.[9] Graphite contains functional groups, such as quinones, that are similar to those in humic substances, a natural electron acceptor for anaerobic respiration in sedimentary environments.[10,11] Although gold is highly conductive, it does not contain such functional groups which conceivably are important in the interaction between electron transport components and electrodes.

However, *S. putrefaciens* represents just one of a wide diversity of microorganisms that might be employed in microbial fuel cells or in biosensors based on microbe−electrode interactions. For example, *Geobacter* species have many potential advantages over *Shewanella* species. *Shewanella* species only incompletely oxidize a limited range of organic acids to acetate, which is inefficient because most of the electrons available in the original fuel remain in the acetate.[3,12] In contrast, *Geobacter* species can completely oxidize organic compounds to carbon dioxide with recovery of >90% of the electrons available in the fuels as electricity.[13−15] *Shewanella* species appear to transfer electrons to anodes via release of a soluble molecule that acts as an electron shuttle,[12] whereas *Geobacter* species establish direct contact with the anode surface and transfer electrons to the anode via one or more redox active proteins.[14,16,17] *Geobacter* species, or close relatives, are the primary organisms that colonize the surface of anodes harvesting electricity from aquatic sediments[13,18,19] and

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swine waste.[20] Studies with the most highly studied *Geobacter* species, *Geobacter sulfurreducens*, have demonstrated that, with a graphite anode, it can produce current densities that are the highest yet recorded by either a pure or mixed microbial culture.[15]

The purpose of this study was to determine if *G. sulfurreducens* could use gold as an anode. The results demonstrate that *G. sulfurreducens* interacts electrochemically with gold almost as effectively as with graphite.

Experimental Section

Gold Electrodes. Gold electrodes were manufactured using the template stripping method,[21] resulting in ultraflat gold surfaces. Silicon wafers (diameter 4 in., thickness 500−550 μm, resistivity: 1−10 Ωcm, crystal orientation: <1 0 0>, type: P, dopant: boron) were purchased from Silicon Quest International, Santa Clara, CA. Glass slides (4 × 4 × 0.2 cm^3) were obtained from the local glass shop and EPO-TEK 377 from Epoxy Technologies, MA. Gold was evaporated with a thermal evaporator at 10^{-6} Torr, to a total thickness of 100 nm, at a rate of 0.2 nm/min.

Fuel Cells. Flow-through "ministacks" with working and counter electrode separated by a Nafion 117 membrane were constructed from commerically available methanol fuel cells as described previously,[15] with the following changes: the anode/working electrode was gold (7.8 cm^2), continuously poised with a potentiostat at a potential of +300 mV vs a Ag/AgCl reference electrode, which was inserted into the working electrode chamber. The counter electrode was a piece of graphite cloth (type GC-14, Electrolytica, Inc., Amherst, NY), 2.4 × 2.4 cm^2, supported by a stainless steel connector. The ministack components were sterilized and assembled as previously described[15] and filled with ethanol (70%). Before use, each ministack chamber was flushed with 1 L of sterile deionized water for over 30 min to remove the ethanol.

Organisms, Media, and Growth Conditions. *Geobacter sulfurreducens*, strain PCA (ATCC 51573)[22] and the mutant in which the gene *pilA* was deleted[23] were obtained from our culture collection and cultured with 10 mM acetate as the electron donor and 40 mM fumarate as the electron acceptor in pressure tubes under strict anaerobic conditions, as previously described.[24] Growth conditions in the ministacks were as previously described,[15] with the following exceptions: freshwater medium[25] contained 0.06 g/L $NaH_2PO_4 \times H_2O$. During recirculation mode, the dilution rate was 8.6 h^{-1} and the initial fumarate concentration was 10 mM. After a switch to flowthrough mode, when current was produced and the working electrode chamber was continuously supplied with medium containing 10 mM acetate, but no fumarate, the dilution rate was 0.86 h^{-1}.

Analytical. For protein quantification cell material was collected by flushing and scraping the biofilm with a pipettor from the electrode surface with 1 mL of sterile isotonic wash buffer (×3),[26] then freeze-dried. Protein was quantified using the bicinchoninic acid assay (Sigma) as previously described.[15] Current was measured with a Power Lab 8SP unit connected to a Macintosh Powerbook G4, and data were logged with Chart 5.4 software (ADInstruments, Mountain View, CA).

Scanning Electron Microscopy. Electrodes were fixed overnight in phosphate buffer (50 mM, pH 7.2) with 2% glutaraldehyde and 0.5 mM sodium azide, cut into pieces (1 × 1 cm^2) under P_i-buffer without glutaraldehyde, and rinsed for 5 min in glutaraldehyde-free phosphate buffer. Samples were incubated for 30 min in phosphate buffer with 1% osmiumtetroxide, and rinsed in phosphate buffer, then in deionized water to remove salts. To dehydrate, the electrode was immersed into increasing concentrations of ethanol (30%, 50%, 70%, 80%, 95%, 100%, 100%, and 100%), 5 min each; the last batch of ethanol was dried before use with activated molecular sieves (4A, 8−12 mesh, JT Baker,). The sample was CO_2-critical point dried from the ethanol transitional solvent. Electrode pieces were mounted on aluminum specimen mounts with Duco cement. The gold layer was electrically connected to the stage using colloidal graphite (TED Pella, Inc., Redding, CA). A 16.5 nm thick gold layer was sputter-coated onto the samples in a Polaron E-5100 sputter coater (3 min at 2.2 kV, 5 mA) with argon at 0.06 Torr, using a gold−palladium target. The samples were observed in a JEOL JSM-5400 scanning electron microscope (SEM) operated at 5 kV.

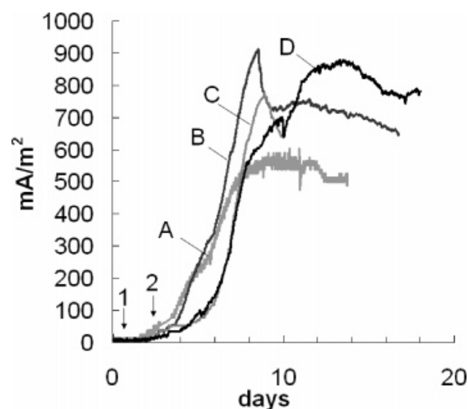

Figure 1. Current generation in mA/m^2 electrode surface area by *G. sulfurreducens* growing on gold electrodes poised at a potential of +300 mV vs Ag/AgCl reference electrode. Four replicates A, B, C, and D, are shown. (1) Inoculation. (2) Switch from recirculating to flowthrough mode.

Confocal Microscopy. Biofilms on gold electrodes were fluorescently stained with the LIVE/DEAD BacLight bacterial viability kit (L7012, Molecular Probes, Inc., Eugene, OR) and examined with confocal laser scanning microscopy, as previously described,[15] with the following exceptions. After disassembly of the ministacks the electrodes were not rinsed before staining, as the biofilm could easily detach. After staining (15 min) and soaking in fresh water medium (5 min), the electrodes were placed upside down on glass cover slips with a droplet of ProLong Antifade agent (P7481; Molecular Probes, Inc., Eugene, OR).[15] A smaller glass cover slip served as a spacer between one side of the gold electrode and the glass coverslip, to create a cavity between electrode and cover slip, where the biofilm was not disturbed and could be examined.

Results

Electricity Production. Current was produced over time when *G. sulfurreducens* was inoculated into chambers with gold anodes (Figure 1). As typically observed in microbial fuel cells,[13,14,27−29] there was a lag period, followed by a rapid increase in current which is associated with growth of the cells on the anode. Assuming that cell growth is exponential, a doubling time of 19.0 ± 3.6 h (mean + standard deviation; $n = 4$) was calculated. This is in the same range as for growth on insoluble Fe(III) oxide (12−24 h).[14] Current stabilized at 0.4−0.7 mA after ca. 6−10 days. This maximum current is comparable to the maximum current previously reported with carbon fiber anodes under the same conditions.[15] The inability to produce more current is probably not due to a limitation in fuel availability because more

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Table 1. Comparison of Current, Specific Electron Transfer (ET) Rate in μmol of Electrons Per Minute and Gram Cell Protein Attached, and Current Density Per Geometric Surface Area of *G. sulfurreducens* on Working Electrodes Composed of Gold or Graphite[a]

material	current (mA)	ET rate (μmol/min·g)	current density (mA/m^2)
flat gold (7.8 cm^2)	0.537 (0.124)	202 (32)	688 (159)
carbon cloth (6.45 cm^2)	2.030 (0.024)	240 (3)	3147 (38)

[a] Values are means of triplicate experiments; standard deviations are given in brackets.

than 70% of the 10 mM acetate flowing into the fuel cell was recovered in the effluent.

Anodes harvested after 14−18 days of incubation were coated with a biofilm that was visible with the naked eye and was red, due to the high abundance of *c*-type cytochromes in *G. sulfurreducens*. Coverage was heterogeneous and the biofilm readily detached from the gold surface, in contrast to *G. sulfurreducens* biofilms on the surface of graphite stick[14,16] or graphite fiber[15] anodes to which the cells tightly adhere.

The cell protein on three gold anodes producing 0.537 ± 0.124 mA was 1.68 ± 0.38 mg. When graphite fiber anodes with comparable geometric dimensions to the gold anodes were substituted for the gold anodes, current production increased nearly 4-fold (Table 1). However, the amount of current produced per gram of cell protein was comparable for the two anode materials. Thus, the increased current with the graphite fiber anodes could be attributed to more biomass on these surfaces. As noted below, the thickness of the biofilms on the carbon fiber and gold anodes was comparable. However, for a given geometric dimension, the highly textured carbon fiber anodes provide much more surface for microbial attachment than the flat gold surface.

Scanning electron microscopy of gold electrodes after 1 month of operation revealed that cells covered much of the gold surface, with cells stacking in on top of each other (Figure 2). Zones where the biofilm had begun to peel off the gold surface were often apparent, consistent with visual observations. As previously observed on graphite anodes,[14] there was no apparent extracellular polysaccharide binding the cells together or to the electrode. In some instances one or two long filaments extended from the cells. It is unlikely that these are the pili reported to be necessary for extracellular electron transfer and biofilm formation[16,23,30] because (1) the filaments on the gold electrodes are much thicker than the 3−5 nm of the pili, (2) *G. sulfurreducens* pili emanate from the side of the cell rather than the ends as seen here, and (3) there are typically many pili emanating from each cell, but only one or two filaments. It is more likely that these filaments are flagella or strands of extracellular material.

However, pili were required for optimal current production with gold anodes. A mutant in which the gene for PilA, the structural pilin protein, had been deleted and thus did not make pili,[23] did not produce current with gold anodes, and cells could not be detected on the gold surface with SEM, even after extended incubation.

Confocal Laser Scanning Microscopy. The procedure for preparing the SEM images can alter the appearance of the biofilm. The treatment with ethanol might wash away constituents, and dehydration might affect structure and thickness.[31] Therefore, the *G. sulfurreducens* biofilms on gold anodes were also examined with confocal laser scanning microscopy. The biofilm that formed after 10 days of growth covered most of the gold anode surface and included pillars up to 12 μm high (Figure 3A). Most of cells in the biofilm stained green, indicating that most of the cells were metabolically active. With longer incubation, the biofilm became thicker, ca. 40 μm (Figure 3B), and more uniform. There was a system of thin channels in the bottom layer of the biofilm (Figure 3B). A high proportion of the cells in these older biofilms stained red, suggesting that they had compromised membranes and might not be metabolically active.

Discussion

These results demonstrate that gold electrodes are a suitable electron acceptor for *G. sulfurreducens*, functioning nearly as well as graphite. This is significant because although gold is highly conductive, it was not clear that microorganisms could use a gold anode as an electron acceptor. Gold does not contain the functional groups, such as quinones, that are present on graphite surfaces and mimic the quinone-containing constituents of natural microbial electron acceptors, such as humic substances.[7] Furthermore, recent studies with the electricity-producing microbe, *S. putrefaciens*, indicated that bare gold was not a suitable anode material for microbial fuel cells.[7]

Gold offers several potential advantages over graphite as an anode material for some applications, as well as basic investigations of microbe−electrode interactions. For example, gold can readily be deposited on a variety of materials and in a diversity of configurations down to the nanoscale. This may be beneficial for applications such as microbially based sensors and small-scale microbial fuel cells. Furthermore, gold offers a highly defined, conductive surface that may be ideal for evaluation of the electrochemical properties of microorganisms growing on anode surfaces.

It may not be surprising that *S. oneidensis* and *G. sulfurreducens* differ in their ability to transfer electrons to gold electrodes because their mechanisms for extracellular electron transfer to anodes appear to be different.[12] Current-producing cells of *G. sulfurreducens* are tightly associated with the anode surface,[14,16] whereas many of the cells in *S. oneidensis* fuel cells are planktonic.[12] Cells of *G. sulfurreducens* closely associated with the anode surface may transfer electrons to the anode via *c*-type cytochromes displayed on the outer cell surface,[17] whereas long-range electron transfer through the biofilm on anodes may proceed via the electrically conductive pili of *G. sulfurreducens*.[16] In contrast, soluble electron shuttles are important for electron transfer to anodes in *Shewanella* species.[12,32] It is beyond the scope of this investigation to determine why an electron-shuttling mechanism would not function with a gold anode, but one possibility is that the electron shuttling molecule(s) are chemically unstable when interacting with a gold surface.

The finding that, per gram of cell protein, *G. sulfurreducens* is nearly as effective in transferring electrons to gold anodes as to graphite suggests that gold does not denature the proteinaceous electron-transfer components necessary for extracellular electron transfer. This might be surprising because some *c*-type cytochromes denature on gold surfaces[9,33−36] and outer-surface *c*-type

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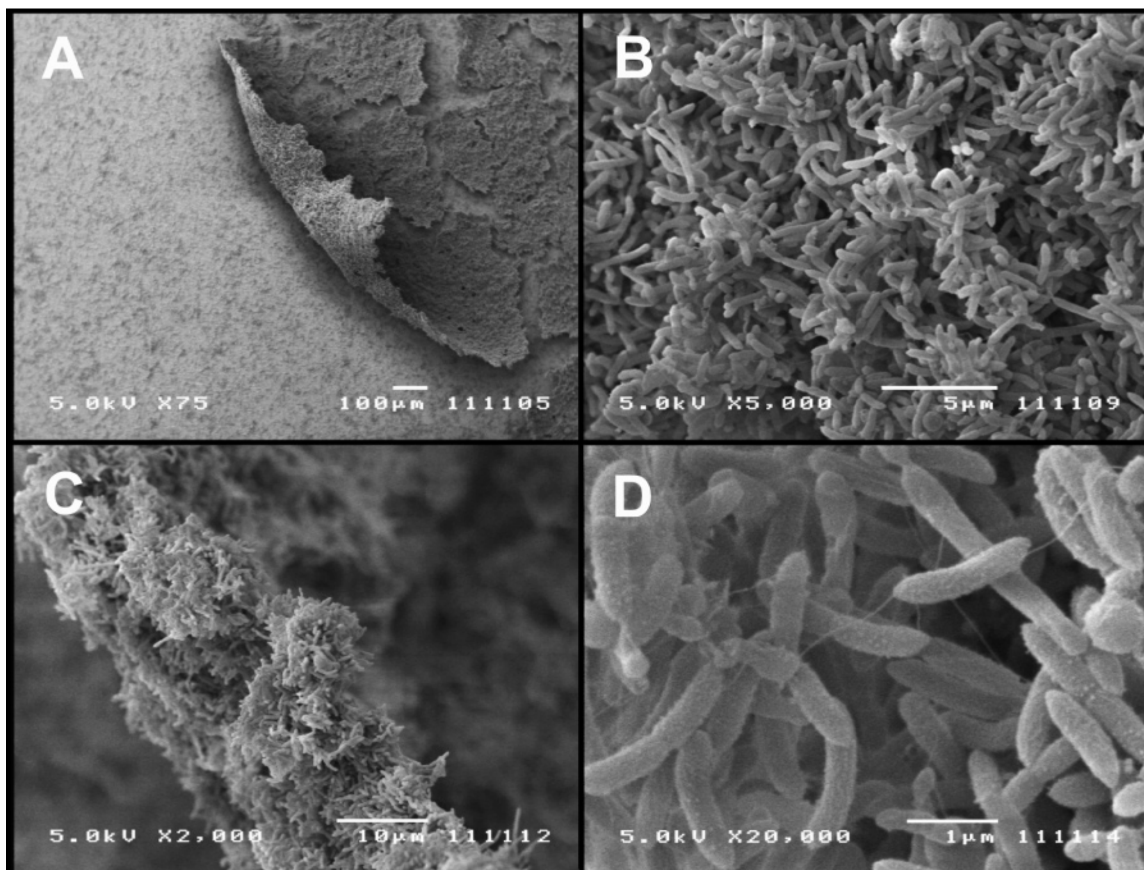

Figure 2. SEM images of *G. sulfurreducens* growing on a gold electrode. Magnification: 75−20 000×. (A) Biofilm attached to the surface, partially peeling off. (B) Closeup of Figure 3A where the biofilm was attached to the electrode surface. (C and D) Closeups of Figure 3A: the edge of the biofilm.

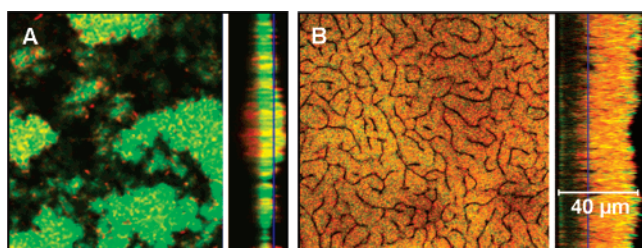

Figure 3. Confocal laser scanning microscope images of *G. sulfurreducens* biofilms on gold electrodes. Magnification: 250×. The large images are top views on the biofilm. The smaller images are orthogonal cross sections with the gold attached side of the biofilm on the left, the outer surface on the right. The vertical blue line is the plane in which the top view image was taken. (A) Biofilm from gold electrode C in Figure 1 after 10 days. (B) Biofilm from gold electrode D in Figure 1 after 18 days.

cytochromes are important in electron transfer to anodes by *G. sulfurreducens*.[17] However, not all electrochemically active biological molecules lose their function in contact with gold, as evidenced by the finding that an organic photochemical cell was constructed with chlorophyll a adsorbed on gold nanoparticles.[37] Unfortunately, none of the outer-surface *G. sulfurreducens c*-type cytochromes have yet been purified so their interaction with gold surfaces cannot yet be readily investigated.

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The finding that the mutant in which the gene for *pilA* was deleted did not produce electricity suggests that the pili are essential for electricity production with gold anodes. This result contrasts with previous results with graphite anodes on which the *pilA*-deficient *G. sulfurreducens* mutant produced low levels of current.[16,17] With graphite anodes, the *pilA*-deficient cells were able to attach to the anode surface, but did not form multiple layer biofilms like the wild type, presumably because long-range electron transfer through the biofilm is not possible in the absence of pili.[16] The total lack of *pilA*-deficient cells on the gold anode suggests that the pili are also required for attachment to the gold anode surface. The reasons for this are not clear.

In summary, the finding that *G. sulfurreducens* can effectively transfer electrons to gold anodes expands the range of materials that can be effectively employed for microbe−electrode electron-transfer reactions. Further studies to understand the mechanisms for this electron transfer to gold are warranted.

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