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Biomagnetic recovery of selenium: Bioaccumulating of selenium granules in magnetotactic bacteria

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Running title: Selenium granule formation in magnetotactic bacteria

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Abbreviation used are: MSGM, magnetic spirillum growth medium; MIC, minimum inhibitory concentration; *M. magneticum* AMB-1, *Magnetospirillum magneticum* AMB-1; TEM, transmission electron microscopy; EDX, energy dispersive X-ray spectrometry.

ABSTRACT

Using microorganisms to remove waste and/or neutralize pollutants from contaminated water is attracting much attention due to the environmentally friendly nature of this methodology. However, cell recovery remains a bottleneck and a considerable challenge for the development of this process. Magnetotactic bacteria are a unique group of organisms that can be manipulated by an external magnetic field due to the presence of biogenic magnetite crystals formed within their cells. In this study, we demonstrated the first account of accumulation and precipitation of amorphous elemental selenium nanoparticles within magnetotactic bacteria alongside and independently to magnetite crystal biomineralisation when grown in a medium containing selenium oxyanion (SeO_3^{2-}). Quantitative analysis shows that magnetotactic bacteria accumulate the highest amount of target molecules (Se) per cell than any other previously reported of non-ferrous metal/metalloid. For example, 2.4 and 174 times more Se is accumulated when compared to Te uptaken into cells and Cd^{2+} adsorption onto the cell surface respectively. Crucially, the bacteria with high levels of Se accumulation were successfully recovered with an external magnetic field. This biomagnetic recovery and effective accumulation of target elements demonstrate the potential for application in bioremediation of polluted water.

IMPORTANCE

The development of a technique for effective environmental water remediation is urgently required across the globe. A biological remediation process of waste removal and/or neutralization of pollutant from contaminated water using microorganism has great potential, but

cell recovery remains a bottleneck. Magnetotactic bacteria synthesize magnetic particles within their cells, which can be recovered by a magnetic field. Herein, we report the first example of accumulation and precipitation of amorphous elemental selenium nanoparticles within magnetotactic bacteria independent of magnetic particle synthesis. The cells were able to accumulate the highest amount of Se compared to other foreign elements. More importantly, the Se accumulating bacteria were successfully recovered with an external magnetic field. We believe magnetotactic bacteria confer unique advantages of biomagnetic cell recovery and of Se accumulation, providing a new and effective methodology for bioremediation of polluted water.

INTRODUCTION

Environmental remediation, a technique of waste removal and/or neutralization of pollutant from a contaminated site, is an attractive field because of the increasing difficulty and importance of pure water acquisition in both developing and industrial countries. Among the various technologies for environmental water remediation, biorecovery of waste using microorganisms has great potential and is an environmentally friendly alternative to conventional techniques such as reclaimation treatment (1–3). Studies of the waste biosorption onto microorganisms and uptake into cells have been well demonstrated, but cell recovery remains a bottleneck in this approach because scale-up of collection methods such as centrifugation and filtration provides a huge logistical and monetary challenge.

Magnetotactic bacteria are unique prokaryotes, recognized by their response to a magnetic field. This is due to the presence of magnetic nanoparticles of Fe_3O_4 or Fe_3S_2 within the cells (4–6).

77 The particle formation occurs within an organelle, called a magnetosome, which is formed along
78 the intracellular filamentous structure (7–9). The magnetosomes confer a magnetic moment to
79 the cells, allowing them to migrate in aquatic environment under the influence of the Earth's
80 geomagnetic field. We have already investigated the use of magnetotactic bacteria for the
81 biomagnetic recovery of toxic and/or valuable metals and metalloid such as Cd (10, 11), Au (12),
82 and Te (13). In these studies, Cd^{2+} and $AuCl_4^-$ were mainly adsorbed onto the cell surface (10,
83 12), while the Te oxyanion (TeO_3^{2-}) was reduced and biomineralized as discrete independent
84 elemental Te nano-crystals within the cells with no incorporation into the magnetite crystals (13).
85 The dual crystallization of tellurium and magnetite by magnetotactic bacteria enabled
86 approximately 70 times more bioaccumulation of the pollutant per cell than cell surface
87 adsorption. Therefore intracellular accumulation of target elements within magnetotactic bacteria
88 offers the most promising system for bioremediation due to the unique advantages of both
89 magnetic manipulation with external magnetic field and of effective target molecule
90 accumulation.

91 Selenium (Se) is a rare element of high use in industry to produce various valuable materials
92 because of its unusual semiconducting and photo-optical physical properties (14). The increased
93 use of Se has led to its rising price and its increase in water contamination, which is in danger of
94 presenting both an ecological and human health risk (15, 16). Therefore, the growing demand for
95 Se in industrial technologies and the increased pollution effects of its byproducts into aquatic
96 environments is rendering the recovery and recycling of this valuable element a very attractive
97 global proposition. In aqueous environments, selenium is generally found as the toxic oxyanions

98 selenate (SeO_4^{2-}, +VI) and selenite (SeO_3^{2-}, +IV). The selenium oxide ions can adsorb
99 extracellularly to the cell surfaces of microorganisms (1, 17). In addition, some microorganisms
100 in the environment possess various strategies of detoxification such as methylation, assimilation
101 as selenoamino acid, and reduction that could provide the potential to effectively accumulate Se
102 within the cell (18, 19).
103 In this study we investigate the minimum inhibitory concentration (MIC) of selenium oxyanion
104 (SeO_3^{2-}) for the magnetotactic bacterium *M. magneticum* AMB-1; the effect of this anion on
105 magnetite crystal synthesis; and if uptaken, whether the Se dopes into the magnetite crystals
106 (similar to the Co and Mn previously reported) (20, 21) or forms discrete crystals/inclusions
107 within the cells (similar to the Te study) (13). Finally, the magnetic recovery of Se using
108 magnetotactic bacteria is investigated.
109
110 **MATERIALS AND METHODS**
111 **Determination of the minimum inhibitory concentration (MIC) of Selenite ion for *M.***
112 ***magneticum* AMB-1 growth.** *M. magneticum* AMB-1 (ATCC700264) (22) was
113 microaerobically cultured in magnetic spirillum growth medium (MSGM) at 28°C as previously
114 described (23). Microaerobic conditions were established by purging the cultures with argon gas.
115 The MIC of selenium for *M. magneticum* AMB-1 in MSGM was determined by growing the
116 cells in various initial concentrations of selenite salt (Na_2SeO_3): 0 (control), 5, 10, 20, 40, 60, 80,
117 100 and 250 µM. The cells were directly counted with a hemacytometer under an optical
118 microscope (Leica DML) after 7 days culture. Additionally the optical density (OD_{600}) was

119 recorded.

120 **Transmission electron microscopy (TEM) and energy dispersive X-ray (EDX) spectrometry**
121 **analyses of *M. magneticum* AMB-1 grown in the presence of SeO_3^{2-}.** Cultured bacterial cells
122 harvested from medium were washed with MilliQ three times and spotted onto 300-mesh
123 Formvar/Carbon coated copper grids (Agar Scientific Ltd). The samples were analyzed by TEM
124 operated at an accelerating voltage of 100 kV (Philips, CM10). High resolution TEM imaging
125 and analysis were conducted on a FEI CM200 field emission gun TEM running at 200 kV
126 equipped with an Oxford Instruments EDX spectrometer and a Gatan Imaging Filter. EDX
127 analysis was conducted for at least 6 crystals in different cells under the same experimental
128 conditions with representative spot data shown.

129 **Se accumulation in *M. magneticum* AMB-1.** To evaluate the amount of uptake and adsorbed
130 SeO_3^{2-} in/onto cells, an atomic absorption spectrophotometer (Shimadzu, AA-6600G) was used.
131 After the cells were collected by centrifugation (or in the case of the magnetic recovery assay,
132 collection by magnetic trap in a glass test tube), the precipitates were washed 3 times with
133 HEPES buffer (pH 7.4), dried and then dissolved with nitric acid solution (0.1N) with heating on
134 in oil bath. After discarding the supernatant, the cells were dissolved with same procedure as
135 described above. The dissolved solutions were quantitatively analyzed by atomic absorption
136 spectrophotometry, using a calibration curve derived from standard solutions. All assays were
137 performed three times.

138 **Magnetic recovery assay of magnetotactic bacteria grown in the presence of selenite ions.**
139 To verify the ability of biomagnetic recovery of *M. magneticum* AMB-1 in the presence of

SeO_3^{2-} using magnetic force, a magnetic cell recovery assay was conducted. The *M. magneticum* AMB-1 wild type strain was harvested at the late logarithmic phase of growth, cells were counted and adjusted to 1.0×10^8 cells/ml of MSGM in the presence of the SeO_3^{2-} at different concentrations (0, 25, 50 and 100 μM). Three milliliters of each sample was then transferred to separate glass test tubes (Diameter: 7 mm, Height: 7.5 cm), each of which was sealed with a rubber cork. Cylindrical neodymium-boron magnets (Diameter: 15 mm, Height: 1 cm) were placed on the exterior of the horizontal centre of each test tube to allow cell recovery to take place. At the designated times (1, 2, 4, 6, 8, 10, 15 and 20h), culture medium was collected by inserting a syringe through the rubber cork and extracting culture medium (20 μl) from around the water surface. A cell count was performed against the extracted culture medium samples. After the magnetic separation, the amount of uptake and adsorbed SeO_3^{2-} in/onto magnetically manipulated cells was evaluated using an atomic absorption spectrophotometer (Shimadzu, AA-6600G). In addition, the magnetically collected cells and Se concentration were measured at the endpoint for further verification.

RESULTS AND DISCUSSION

Effect of SeO_3^{2-} on cell growth and on magnetite biomineralisation in *M. magneticum* AMB-1. The effect of selenium oxyanion (SeO_3^{2-}) on the growth of *M. magneticum* AMB-1 was investigated at various concentrations (Fig. 1). Cells cultured in MSGM containing 0 and 5 μM SeO_3^{2-} showed similar growth rates, with stationary-phase cell densities of approximately 2.2 × 10^8 cells/ml. Cell growth was negatively affected by the increase of SeO_3^{2-} concentration and no

161 cell growth was found at ≥ 250 μM. The MIC of selenium oxyanion for *M. magneticum* AMB-1
162 was determined to be 250 μM under these experimental conditions. The result indicated that
163 SeO_3^{2-} is mildly toxic to this bacteria compared with the other chalcogen, tellurium oxyanion
164 (e.g. MIC = 60 μM) (13). As *E. coli* has a MIC of 400 mM (SeO_3^{2-}), *M. magneticum* AMB-1 is
165 less resistant to this element. Similar observations have been previously found with respect to
166 Co^{2+}, Ni^{2+}, and Cu^{2+} showing approximately 90% less resistance than *E. coli* (20). It is of note
167 that light-orange colors developed during the cell growth in the presence of SeO_3^{2-}. Similar
168 observations were reported in various selenite-reducing bacteria (25, 26). The effect of the
169 chalcogen on magnetite crystal formation in magnetotactic bacteria was also investigated (Fig. 1).
170 The result showed a gradual decrease of magnetosomes with the increase of SeO_3^{2-} concentration
171 but magnetite formation was observed even in the presence of high concentrations (100 μM) of
172 SeO_3^{2-}. In addition, optical microscopy showed that approximately 100% and 70% of cells
173 grown in the presence of 25 μM and 100 μM of SeO_3^{2-} respectively responded to the external
174 magnetic field.

175 **Observation of discrete formation of magnetite crystals and Se granule in *M. magneticum***
176 **AMB-1 grown in the presence of SeO_3^{2-}.** Figure 2a shows representative transmission electron
177 microscope (TEM) images of *M. magneticum* AMB-1 grown in the presence (100 μM) and
178 absence of SeO_3^{2-} in the MSGM medium. Approximately 10 independent spherical granules
179 (30~300 nm diameter) were observed in the cell grown in the presence of SeO_3^{2-} (Fig. 2a), while
180 all cells revealed the presence of the magnetite crystals in a chain structure. The number and size
181 of Se inclusions within the cell increased with increasing initial concentration of SeO_3^{2-} in the

182 medium. In a previous study, we have observed the doping of some metals (Cu, Mn, and Co)
183 into bacterial magnetite crystal under laboratory-controlled conditions (20). However, in this
184 study the elemental mapping showed no signal from Se in magnetite crystals (Fig. 2b). To verify
185 the elemental components in these Se particles, STEM-EDX spot spectra were recorded and
186 showed Se was the only element present (the Cu was from the TEM grid) (Fig. 2b and c). No
187 oxygen was detected, inferring the inclusions are composed of pure elemental Se (0), which
188 seems to be reduced and precipitated from SeO_3^{2-} in the cell. Selenium is a group 16 non-metal
189 (chalcogens), neighbored by sulfur and the metalloid tellurium. Thiosulfate ($S_2O_3^{2-}$), tellurite
190 (TeO_3^{2-}), and selenite (SeO_3^{2-}) are proposed to be taken up by bacteria and reduced to elemental
191 S, Te, and Se, respectively (25, 27, 28). This is supported by the fact that S-globules are present
192 in many microbes, including magnetotactic bacteria (29, 30), and we have also reported the
193 formation of Te nano-crystals in magnetotactic bacteria independent from the magnetosome (13).
194 Here we show for the first time that magnetotactic bacteria uptake, reduce and intracellularly
195 form discrete Se granules independent to magnetosomes, similar to Te crystal precipitation in the
196 same organism (13). The granules were examined by high-resolution TEM with selected area
197 electron diffraction which showed a diffuse pattern, revealing the amorphous Se structure.
198 **Time course measurements of Se accumulation in *M. magneticum* AMB-1.** The time course
199 of Se accumulation in magnetotactic bacteria was measured (Fig. 3). The cell growth and Se
200 accumulation were saturated within 7 days and the Se uptake in cells mainly occurred in the
201 stationary phase (for cells grown in 100 μM of SeO_3^{2-}). Under this condition, 68.1% of the initial
202 Se (100 μM) was accumulated by the cells, which accounts to 6.6×10^8 Se atoms per cell. In the

203 case of Te accumulation found in the previous study, the most effective condition revealed that
204 2.7×10^8 Te atoms were accumulated per cell, which indicates that 2.4 times more Se is
205 accumulated than Te. Furthermore, surface hexa-histidine expressing modified AMB-1 cells
206 have previously been shown to adsorb Cd^{2+} onto these sites on the cell surface, showing the
207 adsorption of 3.8×10^6 metal ions. Therefore, 2.4 and 174 times more Se was accumulated when
208 compared to Te in cell and Cd^{2+} adsorption onto cell surface. These results highlight the greater
209 loading of elemental Se into AMB-1 cells than any other metalloid or non-ferrous metal.

210 **Biomagnetic recovery of SeO_3^{2-} using *M. magneticum* AMB-1.** Magnetotactic bacteria
211 harboring our target element (Se) for recovery can be manipulated and isolated by an external
212 magnetic field, significantly magnifying the bioremediation potential of these cells for targeted
213 recovery from polluted water environments. Herein, biomagnetic recovery of magnetotactic
214 bacteria grown in the presence of SeO_3^{2-} was conducted. The result shown in Fig. 4 revealed that
215 almost all cells grown in 25 μM SeO_3^{2-} were successfully recovered within 8 hours. The time for
216 magnetic recovery of cells gradually increased with increasing concentration of SeO_3^{2-}. This
217 seems to be the result of the decreasing quantities of magnetite under higher Se concentration
218 conditions (Fig. 1). However, even in the presence of 100 μM SeO_3^{2-}, approximately 80% of
219 magnetotactic bacteria were magnetically recovered within 20 hours. To confirm the
220 biomagnetic recovery of Se, the amount of Se from magnetically recovered harvested cells was
221 measured and revealed 3.6×10^8 Se atoms per cell recovery. Though some Se was lost during the
222 recovery process (3.0×10^8 Se atoms after recovery), the result clearly shows that magnetotactic
223 bacteria could be applied in biomagnetic recovery of Se from SeO_3^{2-} containing water. We note

224 that a more effective recovery could be established by process optimization (e.g. cell number,
225 vessel size and magnetic force enhancement).

226 Current genetic and environmental microbiological research shows that magnetic particle
227 production within bacteria occurs across a diverse group of bacterial species. In fact, the genetic
228 region corresponding to magnetosome formation, called magnetosome island (MAI), is found
229 within microbes spread across the phylogenetic tree. As *M. magneticum* AMB-1 does not show
230 strong resistance to SeO_3^{2-} (Fig. 1), a magnetotactic bacterial species with higher tolerance and
231 effective accumulation of target molecule could be found and used to improve the biomagnetic
232 recovery; identified either from environments local to the bioremediation site or through
233 evolving conditions to those similar to the polluted environment for a range of candidate
234 magnetotactic bacteria. In addition, recently, magnetosome formation was enabled in another
235 bacterial species by artificially transferring key genetic regions of the MAI into the host
236 organism (31). Therefore, the induction of magnetosome formation within known bacteria
237 showing high resistance to target element is another promising approach to improve the
238 biomagnetic recovery efficiency.

239 In conclusion, in this study we showed the first account of amorphous elemental Se particle
240 formation from the reduction of SeO_3^{2-} within the magnetotactic bacterial cell, completely
241 independent of the crystallization of magnetite within the cells' magnetosomes. The cells were
242 accumulated the highest amount of Se compared to any other foreign elements. For example, 2.4
243 and 174 times more Se was accumulated as compared to Te in cells and Cd^{2+} adsorption onto cell
244 surfaces. Importantly, the Se accumulating bacteria were successfully recovered with an external

magnetic field. Therefore, we believe magnetotactic bacteria have the unique advantage of biomagnetic cell recovery, providing a new effective methodology for bioremediation of polluted water and additional potential to utilize the pollutant product for further material applications.

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M. T., A. A., S. B., S. S., and T. M. conceived and designed the experiments. M. T., W. K., R. B., N. H., and S. S. performed the experiments. All authors analyzed the data. M. T., A. A., S. S., and T. M. wrote the paper. All authors have no conflict of interest directly relevant to the content of this article.

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343 **FIGURE LEGENDS**

344 **Fig. 1. Tolerance of *M. magneticum* AMB-1 to SeO_3^{2-} and magnetite nano-particle**
345 **synthesis.**

346 The number of cells (□) and magnetite crystals (●) grown in different concentrations (0, 5, 10,
347 20, 40, 60, 80, 100, and 250 μM) of SeO_3^{2-} were directly counted. To evaluate the number of
348 magnetite within the cells, over 50 cells randomly selected were manually counted. Error bars
349 show SDs.

350

351 **Fig. 2. Transmission electron micrographs, and STEM-EDX analyses for magnetite and**
352 **Se within magnetotactic bacteria.**

353 (a) TEM micrographs of magnetotactic bacteria grown i) in the presence of SeO_3^{2-} (100 μM) and
354 ii) in its absence. Characteristic intracellular granules were indicated with arrows. Scale bar
355 indicates 100 nm. (b) TEM image and STEM-EDX maps of Se, Fe, and O taken using a probe
356 size of approximately 5 nm. (c) Spot EDX spectra of *i and *ii in b) as a representation of Se
357 and magnetite. The Cu signal is from cupper TEM grid.

358

359 **Fig. 3. SeO_3^{2-} removal during magnetotactic bacterial cell growth.**

360 SeO_3^{2-} removal using magnetotactic bacteria (○) and cell growth (◆) was evaluated in the
361 presence of 100 μM SeO_3^{2-} for 7 days. The average values from three independent experiments
362 were obtained. Error bars show standard deviations.

363

364 **Fig. 4. Magnetic recovery assay of Se granule-containing *M. magneticum* AMB-1.** The
365 percentage of recovered cells is calculated from the initial cell numbers (1.0×10^8/ml) by
366 counting the number of dispersed cells left within the culture medium. In addition, the number of
367 cells recovered by magnetic force was also verified by counting the cells recovered at the end
368 points. *M. magneticum* AMB-1 was cultured and assayed with the respective concentrations of
369 SeO_3^{2-} (SeO_3^{2-} concentration = 0 μM (control) (■), 25 μM (○), 50 μM (▲), and 100 μM (◇)).
370 The average values from three independent experiments were obtained. Error bars show standard
371 deviations.

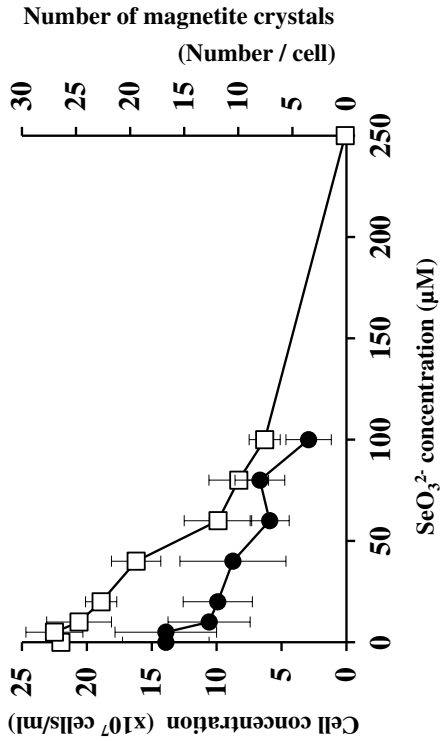

Figure 1

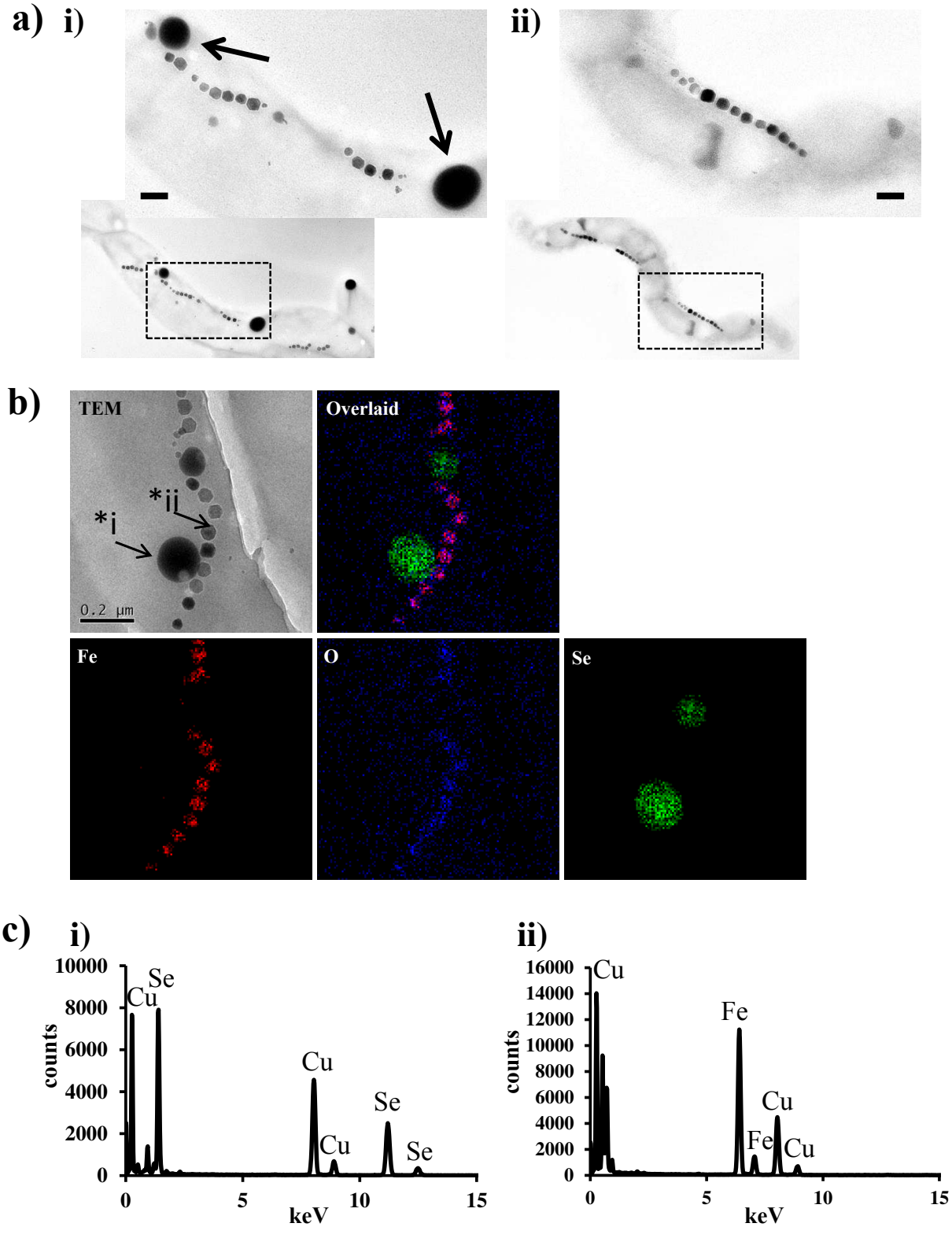

Figure 2

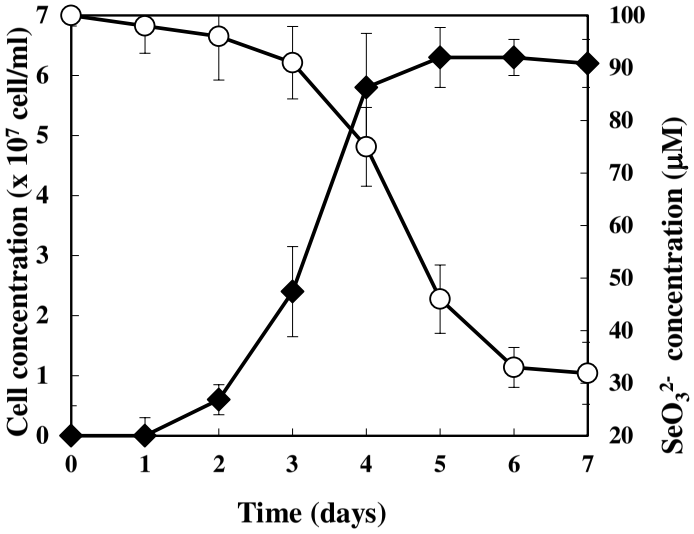

Figure 3

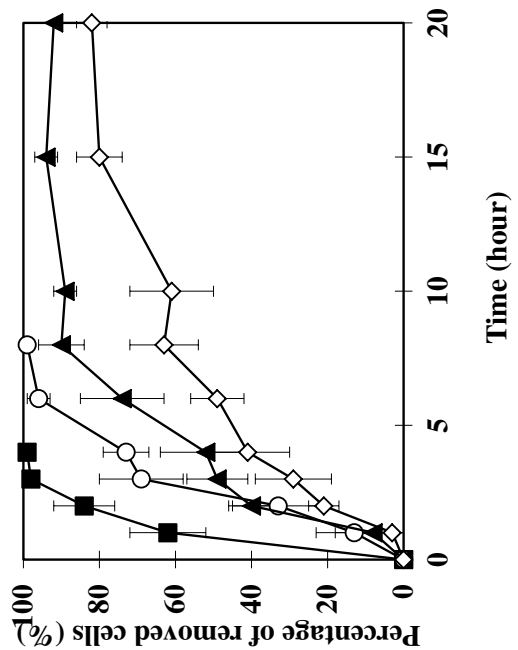

Figure 4