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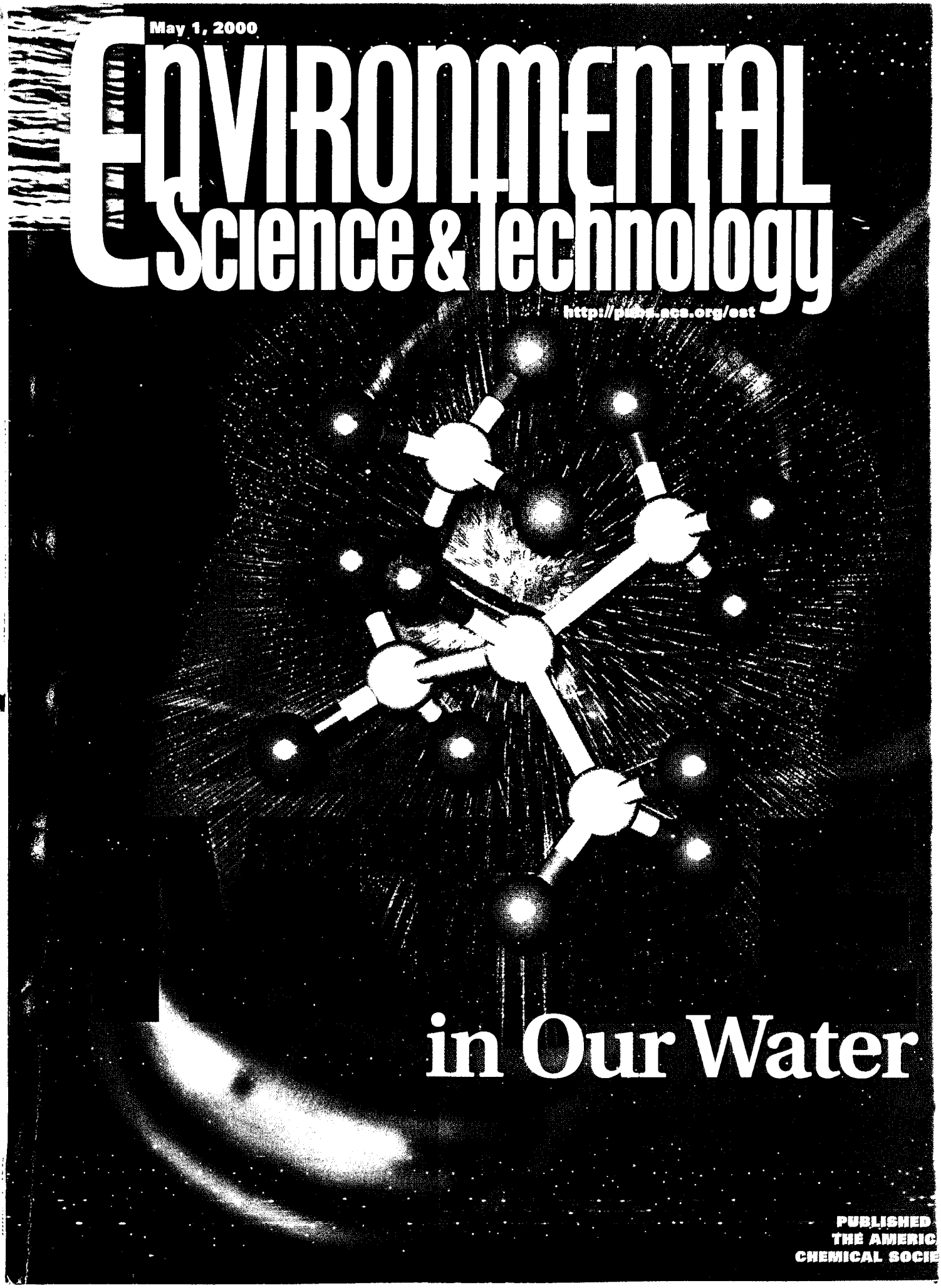

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Bacillus lichenformis γ-Glutamyl Exopolymer: Physicochemical Characterization and U(VI) Interaction

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Complexation by microbially produced exopolymers may significantly impact the environmental mobility and toxicity of metals This study focused on the conformational structure of the bacterial exopolymer, γ-D-poly(glutamic acid) and its interactions with U(VI) examined using ATR-FTIR spectroscopy Solution pH, polymer concentration, and ionic strength affected the conformation of the exopolymer, and U(VI) binding was monitored At low pH, low concentration, or low ionic strength, this exopolymer exists in an α-helical conformation, while at high pH, concentration, or ionic strength the exopolymer exhibits a β-sheet structure The change in exopolymer conformation is likely to influence the number and nature of exposed surface functional groups, sites most responsible for metal complexation We found the polyglutamate capsule binds U(VI) in a binuclear, bidentate fashion, in contrast the glutamate monomer forms a mononuclear, bidentate complex with U(VI) The apparent polynuclear binding of U(VI) may induce β-sheet structure formation provided the U(VI) concentration is sufficiently high

Introduction

Metals and radionuclides are the most common binary contaminant mixture at DOE waste sites (*1*), and remediation of contaminated soils, sediments, and waters is a tremendous challenge facing our nation Much research has been carried out in an attempt to reduce the environmental risks of metals and radionuclides by means of biogeochemical processes (*2–5*) Research has substantiated that microorganisms play a critical role in immobilizing metals and radionuclides in the subsurface environment The concentration of dissolved species may be decreased through adsorption or precipitation (*6–10*) On the other hand, interactions with bacteria may enhance the mobility of metals and radionuclides in soils and sediments (*11–16*) Dissolved macromolecules (e g humic acids or biopolymers produced by microbes), in particular, bind metals and radionuclides, allowing them to be transported along with these much more mobile carriers (*16–21*) An understanding of metal interactions with biomacromolecules will provide a basis for ecological risk assessment and for developing engineered systems for enhancing or retarding mobilization of radionuclides and other metals in contaminated soils and sediments

One predominant type of biomolecule in the environment is the bacterial exopolymer These polymers are produced by microbes to protect against desiccation, to anchor cells to surfaces, and to help bacteria evade predators (*19*) Exopolymers interact with a wide variety of toxic metals in the environment and, because of their charged surface functionalites, can form complexes with them This metal binding may significantly affect the fate and transport of toxic metals in subsurface environments (*16*) For example, an exopolymer produced by a freshwater-sediment bacterium has a high binding affinity for Cu(II) (*22*) Chen et al (*17*) demonstrated that the adsorption of Cd(II) and Pb(II) by sediments was reduced by more than 90%, while exopolymers were introduced to the system Exopolymers have been found to have a retardation factor orders of magnitude lower than those for Pb(II) and Cd(II), (*18*) suggesting that exopolymers themselves and exopolymer–metal complexes can be transported readily in soils and sediments

Bacillus lichenformis, a common soil microbe, produces a poly(γ D-glutamic acid) exopolymer (γ-PGA) γ-PGA was first described in 1937 and has been extensively studied in terms of its synthesis, biochemistry, and production under various environmental conditions (*23–28*) This exopolymer is produced by other *Bacillus* species, such as *B subtilis* The γ-PGA molecular structure contains amide linkages between the α-amino and α-carboxylic acid functional groups (*28*), leaving the γ-carboxylate group available for cation binding McLean et al (*29*) reported that γ-PGA isolated from *B lichenformis* binds a variety of metals including Ni(II) Cu(II) Mn(II) Al(III), and Cr(III) It was also found that the oxidation state of Fe significantly influences Fe-exopolymer binding Under aerobic conditions the addition of Fe(III) to an exopolymer solutions resulted in the formation of a rust precipitate, whereas addition of Fe(II) did not yield a precipitate or induce a color change (*30*) While McLean and co-workers examined heavy metal interactions with PGA, we are unaware of any studies that have investigated complexation of actinides with this exopolymer

Bacterially produced exopolymers, as most charged or polar macromolecules, can exist in various configurations, depending on solution conditions Important factors that can change the overall conformation and local structure of biopolymers include temperature, pH, capsule concentration, and background electrolytes (*31*) These factors affect the orientation of biopolymer functional groups, determining the apparent (effective) charge, metal binding properties and, subsequently, environmental mobility A preliminary study suggested that conformational changes in the exopolymer produced by *B lichenformis* occurred, but no detailed studies were performed and the nature of the changes was not reported (*32*)

In this study, we have used attenuated total reflectance Fourier transform infrared spectroscopy (ATR) FTIR (*33*) to examine the conformational structure of bacterially produced exopolymer as affected by pH, polymer concentration, and ionic strength Because uranium is a predominant contaminant at DOE sites (*1*), U(VI) complexation by the exopolymer was also examined

Materials and Methods

Chemicals and Solutions Water used throughout was deionized using a Millipore water purification system operated at an impedance of 18 Ohms Glutamic acid, NaCl, $NaNO_3$, sodium azide and Pronase were obtained from Sigma

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and used without purification Uranyl nitrate hexahydrate was obtained from Alpha-Aesar and used without purification Glacial acetic acid was obtained from Fisher and used without purification

Organism and Strain Maintenance. *Bacillus licheniformis* was provided by Dr R J C McLean (Southwest Texas State University) Highly mucoid colonies that produced large quantities of γ-PGA were selected from plates of medium E (*34*) and incubated at 37 °C overnight Selected colonies were grown in liquid medium E with shaking (250 rev min^{-1}) at 37 °C overnight Freezer stocks were made by transferring aliquots of the culture to cryovials and freezing with liquid nitrogen

Optimization of Exopolymer Production by *B licheniformis* A frozen culture of *B licheniformis* was thawed at room temperature and inoculated into a 250-mL flask that contained 100 mL of Medium E The flask was incubated for 24 h at 37 °C Medium E agar plates were spread with 0 5 mL of the culture and incubated at 30 and 37 °C Cells and exopolymers were harvested with 0 1 M NaCl at selected times to determine exopolymer yield The harvested suspensions were centrifuged for 15 min at 16 000*g* Following centrifugation the supernatants were stored at 4 °C for determination of exopolymer molecular weight distribution Colony forming units/mL (CFU/mL) were determined by suspending the pellets, measuring absorbance at 600 nm, and calculating numbers from a previously prepared standard curve of absorbance vs CFU/mL.

Characterization of the Exopolymer Produced by *Bacillus licheniformis*. The molecular weight of the exopolymer was qualitatively estimated using SDS tris-glycine gel electrophoresis (SDS-TGGE) and quantitatively determined using gel permeation chromatography (GPC) SDS-TGGE was done according to Yamaguchi et al (*35*) Briefly, an exopolymer suspension of 0 2 mL was mixed with 0 2 mL of sample buffer (4% SDS, 20% glycerol, 0 125 M Tris, 0 005% bromophenol blue, and pH 6 8) The mixture was heated for 2 min at 90 °C Electrophoresis was run at 94 V for approximately 2–3 h Exopolymer staining was accomplished with alcian blue (0 5% in 3% acetic acid) Protein standards were stained with Coomassie brilliant blue R-250 The relative molecular weights of exopolymers were estimated using protein standards

The average molecular weight (M_w) of γ-PGA was measured with gel permeation chromatography (GPC), essentially by the method of Birrer et al (*34*) A Rainin HPXL HPLC (Rainin Instrument Company, Inc , Emeryville, CA) was used The column employed was a Shodex OH pak SB805 HQ (Phenomenex) maintained at 37 °C with a CH-500 column heater (Eppendorf, Madison, WI) The detector was the 1770 differential refractometer (BioRad Laboratories, Hercules, CA) Pullulan standards (set P-82) with narrow polydispersities were purchased from Phenomenex Standards with concentration of 1 g/L were used to construct a calibration curve, from which the molecular weights of γ-PGA were calculated The mobile phase, 0 2 M sodium nitrate with 0 02% (w/v) sodium azide, was adjusted to pH 4 0 using glacial acetic acid The γ-PGA solution was filtered through 0 45 μm GHP Acrodisc filters (Gelman) prior to injection to the GPC system The mobile phase flow rate was maintained at 1 0 mL per min, and the injection volume was 100 μL

Preparation and Purification of γ-D-Poly(glutamic acid) (γ-PGA) The procedure described here was modified after McLean et al (*29*) A freshly prepared culture was inoculated into large agar (Medium E) plates and incubated at 37 °C for 12 h Cells and exopolymer were harvested with 0 1 M NaCl The cell-exopolymer suspension was centrifuged for 15 min at 16 000*g* The supernatant was stored at 4 °C The pellets were suspended in 0 1 M NaCl, and exopolymer was sheared from the cells using a Waring blender (2 min at 4 °C) The resulting suspension was centrifuged, and the supernatant was combined with the first supernatant Cold ethanol was added to the supernatant to obtain a 1 4 v/v supernatant ethanol mixture from which exopolymer precipitated after 12 h at −20 °C Following filtration through a Gelman Supro-200 0 2 μm membrane, the exopolymer precipitate was suspended with a minimal volume of 0 1 M NaCl This suspension was dialized in a metal, sulfide, and glycerol-free cellulose ester dialysis membrane (50 000 MWCO) (Spectrum Laboratories, Inc , Laguna Hills, CA) against 0 5 M NaCl for 24 h at 4 °C to remove residual ethanol After dialysis, ribonuclease (20 μg/mL), deoxyribonuclease (20 μg/mL), and 10 mM $MgCl_2$ were added to remove nucleic acids Additionally 0 02% (wt/vol) sodium azide was added to inhibit bacterial exopolymer degradation The solution was incubated for 1 h at 37 °C and repeated to ensure complete nucleic acids removal Pronase (5 μg/mL) was added, and the solution was incubated for 1 h at 37 °C and repeated to remove proteins The exopolymer was then dialyzed against varying concentrations of NaCl 0 5 M NaCl, pH 2, 0 5 M NaCl, pH 10, and 0 1 M NaCl, pH 5 5 solutions once and four times with deionized water at 4 °C Solutions were changed every 24 h The purified exopolymer solution was stored at 4 °C The concentration of purified exopolymer solution was measured by lyophilizing purified exopolymer and determining the weight of the dried material

Spectrophotometric Measurements Optical absorption spectra were obtained using a Perkin-Elmer Lamda2 UV–vis spectrophotometer (Bodenseekerk, Germany) Suprasil quartz cells (Fisher Scientific) were used for all determinations Spectra were collected over the UV–vis range in a double beam mode with a resolution of 1 nm After each measurement, the solution pH was determined When necessary, suspensions were filtered through 0 45 μm GHP Acrodisc filters (Gelman)

Infrared spectra of solutions and suspensions were recorded using the Magna-IR 560 FTIR spectrometer equipped with a deuterated triglycine sulfate (DTGS) detector (Nicolet Instrument Corporation, Madison, WI) An attenuated total reflectance (ATR) CIRCLE open-boat type cell (Spectra Tech, Inc , Shelton, CT) was used to measure 5 mL liquid samples The internal rod-shaped zinc selenide crystal has a diameter of 6 35 mm and is designed to have an average angle of light incidence of 45° and 16 reflections The cell was covered to prevent evaporative loss ATR-FTIR spectra were collected from 4000 to 700 cm^{-1} with 1024 scans at a resolution of 4 cm^{-1}, unless otherwise indicated All final ATR-FTIR spectra were obtained by subtracting the reference spectrum (background buffer or deionized water) from the sample spectrum Reference and sample spectra were ratioed against an empty cell spectra to accurately remove H_2O bands

Samples for ATR-FTIR spectroscopy included UO_2^{2+}, glutamate, bacterial exopolymer, and U(VI)–glutamate and exopolymer mixture solutions in H_2O or 0 1 M NaCl Solutions were adjusted to the desired pH prior to spectrum collection After each measurement, the pH of the solution was measured When necessary, suspensions were filtered through 0 45 μm GHP Acrodisc filters (Gelman)

Results

Exopolymer Production, Characterization, and Stability Exopolymer production was low during the first 12 h at both 30 °C and 37 °C but increased dramatically after 18 h at 37 °C and after 24 h at 30 °C For both time periods (18 h at 37 °C and 24 h at 30 °C), slight exopolymer degradation was observed by gel electrophoresis Beyond these times, exopolymer degradation was significant We selected 12 h incubation at 37 °C to produce high quality exopolymers, with a M_w of approximately 800 000 mol g^{-1} as determined with GPC The purified exopolymer was stable over 6 months

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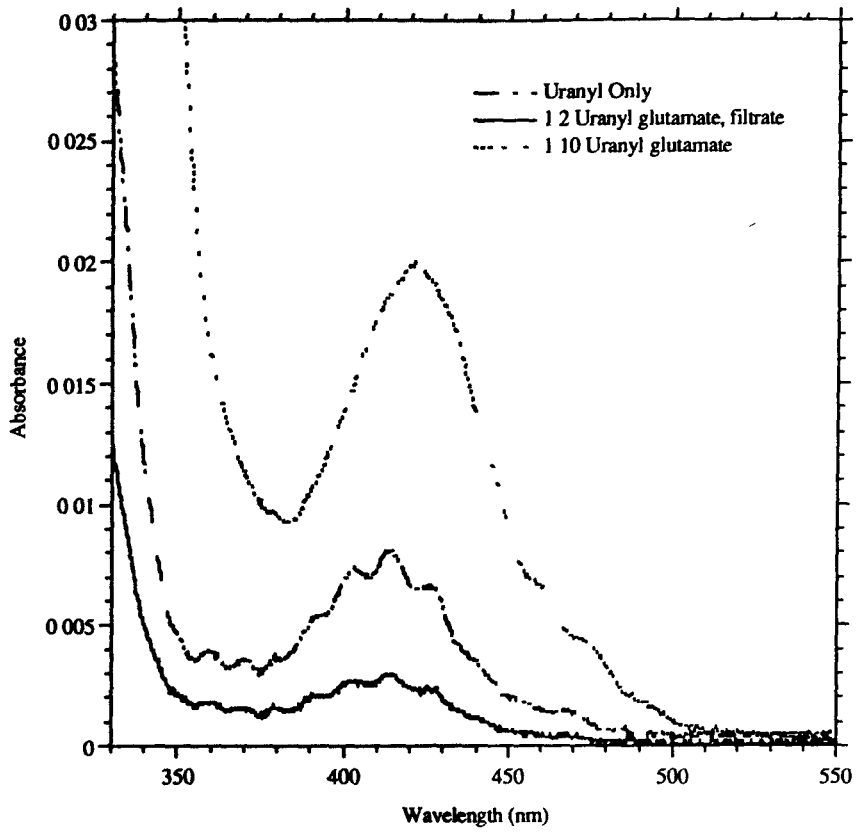

FIGURE 1 Optical absorbance spectra of U(VI)-exopolymer at varying U(VI) exopolymer ratios 1 mM exopolymer (based upon glutamate subunit), 0 1 M NaCl, pH 3

at 4 °C and for at least 10 d at 15 ° C At room temperature (~24 °C), the average M_w of the exopolymer decreased to approximately 700 000 mol/g after 24 h but then remained unchanged afterward up to 8 d Unpurified exopolymer was rapidly degraded even if stored at 4 °C

The purified exopolymer displayed tolerance to acid A 0 1 M HCl solution did not cleave or otherwise degrade the exopolymer, while a 1 M HCl solution caused significant degradation of the polymer after 24 h exposure at 4 °C (final M_w = 200 000 mol/g) as determined by GPC

Optical Spectra of U(VI) Exopolymer Exopolymer–U(VI) complexes were examined using UV–vis spectroscopy For all UV–vis measurements, the ratio of U(VI) to exopolymer was normalized to moles of U(VI) to moles of glutamate monomer and will be expressed as uranyl glutamate monomeric unit A solution of 1 0 mM uranyl nitrate at pH 3 was prepared, and its optical absorbance spectrum (characteristic of free UO_2^{2+} , λ_{max} = 413 5 nm and ϵ = 7 95 Abs/mol•cm) is shown in Figure 1 (*36, 37*) Solutions were then prepared with the same uranyl concentration, the same pH, and varied exopolymer concentrations (1 1–1 10) At the ratios of 1 1 to 1 5 a white precipitate immediately formed, at the ratio of 1 10 all of the product remained in solution The precipitates were removed from solution, and the absorbance spectrum of the filtrates were measured to determine the amount of uranyl bound by the exopolymer For example, the 1 2 uranyl to glutamate filtrate had an absorbance corresponding to about one-third (~36%) of the uranyl remaining in solution and about two-thirds bound by the exopolymer, suggesting a ratio of three glutamate subunits to each uranyl in the precipitate formed under these conditions (assuming all of the exopolymer precipitated) When the ratio was increased to 1 10, no precipitate was observed, and a spectrum was observed with a λ_{max} at 420 8 nm and a ϵ_a = 20 Abs/mol cm, characteristic of a uranyl carboxylate complex. These data confirm that uranyl is complexed by the exopolymer and that resulting complex is only soluble when the polymer is present in a ≥10-fold excess (based on monomer)

ATR-FTIR Measurement U(VI) Solution The infrared spectrum of uranyl as a function of pH was measured to determine conditions most appropriate for exopolymer binding studies The characteristic band for UO_2^{2+} at pH 1 84 was 962 cm^{-1} (Figure 2), attributed to the asymmetric stretch of uranyl As solution pH is increased, hydrolysis of U(VI) was observed At pH 3 69, a second band at 947 cm^{-1} was present, corresponding to the first hydrolysis species (Figure 2) At pH 4 11, the solution of 30 mM U(VI) formed precipitates, and only one band at 942 cm^{-1} was observed for the suspension After filtration of this suspension, the supernatant showed two bands, i e , 962 and 942 cm^{-1}, with a much lower absorptive intensity This suggests that at least two species (e g , free UO_2^{2+} and hydrolyzed $(UO_2(OH))_2^{2+}$ were detectable in the supernatant The IR bands and hydrolysis behavior are consistent with previously reported uranyl hydrolysis studies (*38, 39*) and confirmed that use of pH 3 solutions for further experiments would avoid competition with hydrolysis or the formation of mixed hydroxo, glutamate complexes

U(VI)–Glutamate Complexation Aqueous glutamate was examined first to obtain the spectrum of uranyl bound by the subunit of the exopolymer and enable comparison of results for the monomer and the large microbially produced polymer At pH 3, the ATR-FTIR spectral bands for aqueous glutamate (Figure 3) can be assigned as follows The 1735–1696 cm^{-1} region corresponds to the γ-carboxylate C=O and the 1225 cm^{-1} band to C–OH stretches (Glutamate γ-carboxylate has a pK_a of 4 4 and thus is protonated at pH 3) The band between 1650 and 1620 cm^{-1} not only corresponds to the asymmetric deformation of $-NH_3$ but also can be attributed to the asymmetric stretching of the deprotonated

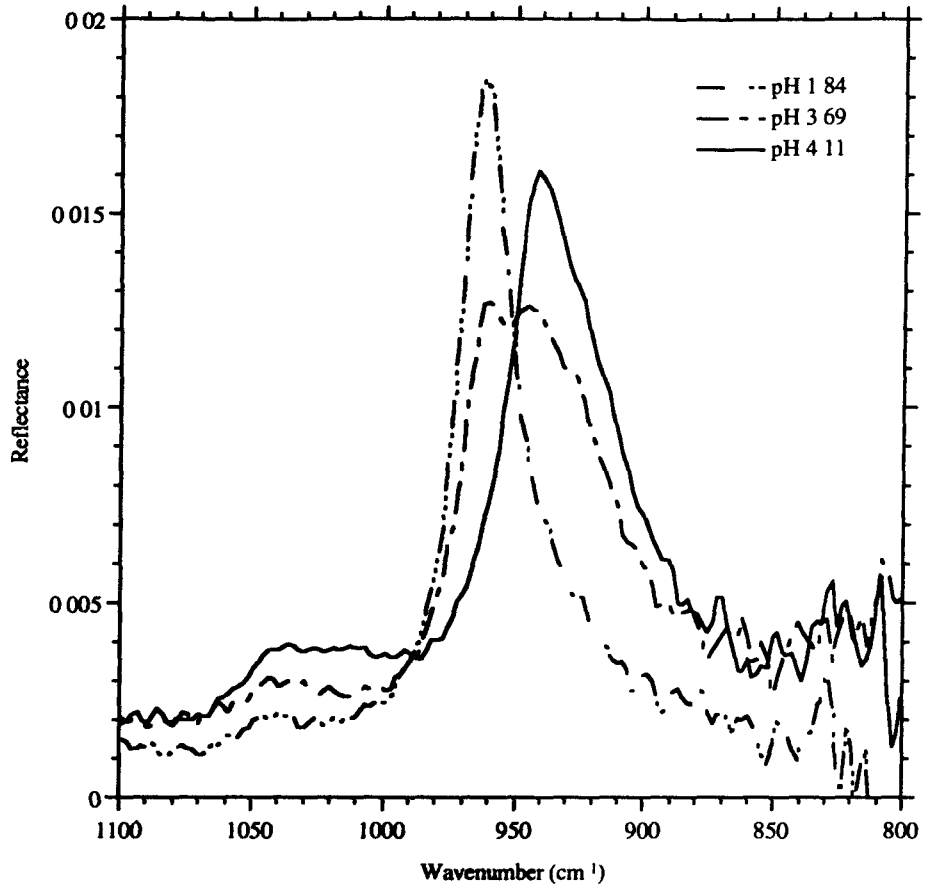

FIGURE 2. ATR-FTIR spectra of aqueous U(VI) in 0 1 M NaCl as a function of pH

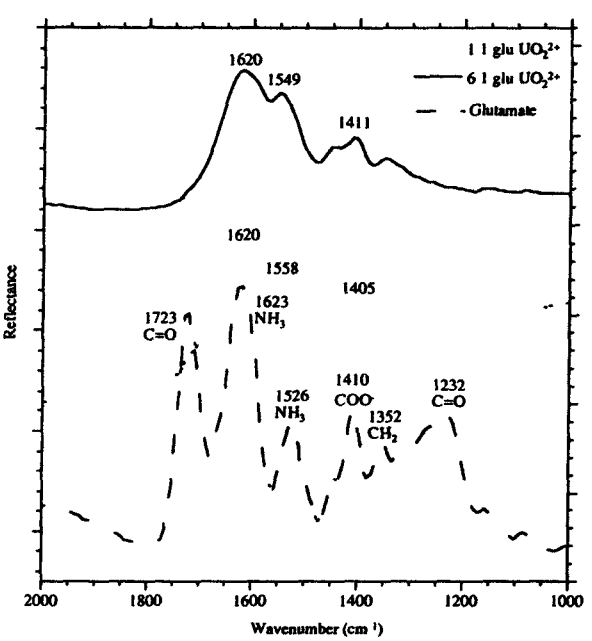

FIGURE 3. ATR-FTIR spectra of U(VI)-glutamate as a function of U(VI) glutamate ratios, 0 1 M NaCl, pH 3

α-carboxylate group (−COO), which has a pK_a value of 2 2 The 1526 cm^{-1} band corresponds to the $-NH_3$ symmetric deformation The band at 1410 cm^{-1} is due to the symmetric stretching of the deprotonated α-carboxylate group The bands at 1526 and 1352 cm^{-1} correspond to the $-NH_3$ and $-CH_2$ deformation, respectively (*40*)

When U(VI) was added to the glutamate solution the γ-carboxylate stretches in the 1735−1696 cm^{-1} region and at 1225 cm^{-1} disappeared, indicating ligand complexation of the metal ion (Figure 3) At the 1 1 ratio of U(VI) glutamate, the symmetric stretch of the carboxylate group shifted to 1405 cm^{-1} At the 6 1 ratio, both the asymmetric and symmetric stretches of the deprotonated carboxylate group shifted, the former to 1549 cm^{-1} and the latter to 1411 cm^{-1} (Figure 3)

Exopolymer Solution The exopolymer in deionized water and in 0 01 M NaCl solution exhibited a band at 1650 cm^{-1}, which is attributed to the amide I stretch (Figure 4) At an ionic strength of 0 1 M NaCl, the amide I band shifted to 1640 cm^{-1} When the ionic strength was increased to 0 5 M, the amide I band shifted further to 1635 cm^{-1} The amide II band at 1558 cm^{-1} did not change with ionic strength ranging from deionized water to 0 5 M NaCl (Figure 4) The two bands at 1720 and 1233 cm^{-1} corresponding to the protonated γ-carboxylate group stretchings of the exopolymer did not shift with changing ionic strength

The amide I band also changes with exopolymer concentration as shown in Figure 5 In 0 01 M NaCl at pH 3 0, the exopolymer (5 mM as glutamate) showed a band at 1650 cm^{-1} (amide I) At a 5-fold higher concentration, however, the amide I band was at 1635 cm^{-1} (Figure 5) The amide II band was at 1558 cm^{-1} for both exopolymer concentrations

The effect of solution pH on exopolymer conformation in 0 01 M NaCl was examined As the solution pH was increased from 3 0 to 5 45, the amide I band shifted from 1650 cm^{-1} and two other bands appeared at 1585 and 1405 cm^{-1} The 1585 cm^{-1} band may be related to the vibrations of $-NH_2$ deformation and the asymmetric stretch of the deprotonated carboxylate group (−COO), and the 1405 cm^{-1} band corresponds to the symmetric stretch of −COO (*41*) These two bands were more evident at pH 9 70 (Figure 6)

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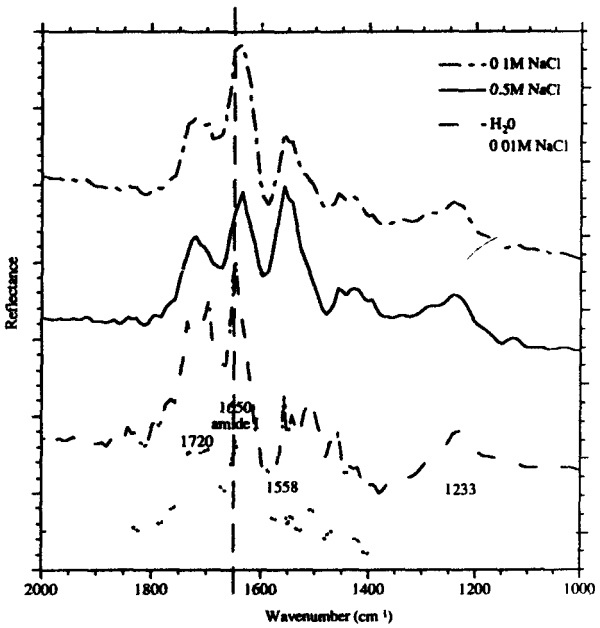

FIGURE 4 γ-PGA ATR-FTIR spectra as a function of ionic strength

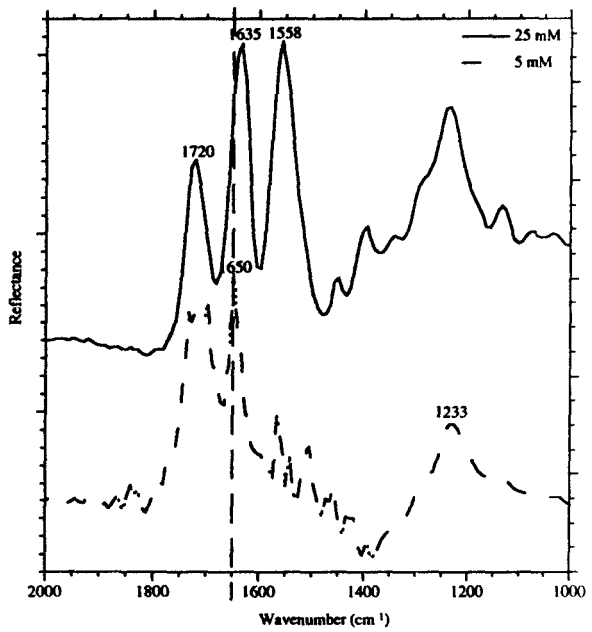

FIGURE 5 γ-PGA ATR-FTIR spectra as a function of polymer concentration, 0 01 M NaCl, pH = 3

U(VI)–Exopolymer Complex. Uranyl ions complex with exopolymer by substituting H^+ ions on protonated carboxylate groups, causing changes in IR frequencies of exopolymer The 1239 cm^{-1} band corresponding to the protonated carboxylate stretch disappeared with addition of U(VI) to the exopolymer solution (Figure 7) Addition of U(VI) did not change the frequencies for stretches of the deprotonated or U(VI)-complexed carboxylate groups, i e , 1558 cm^{-1} for the asymmetric and 1396 cm^{-1} for the symmetric However, at the 2 1 ratio of U(VI) exopolymer (as glutamate), the amide I stretch shifted from 1650 cm^{-1} to 1635 cm^{-1} (Figure 7)

Discussion

Characteristics of the Exopolymer Molecular Weight and Stability The exopolymer, γ-PGA, produced by *B licheniformis* was of varied molecular weight ranges and qualities under a range of temperatures and incubation times We

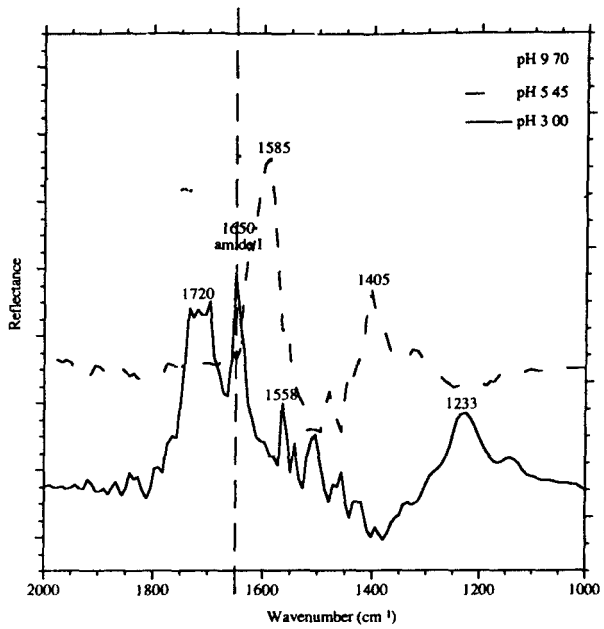

FIGURE 6 γ-PGA ATR-FTIR spectra as a function of pH polymer concentration = 5 mM, 0 01 M NaCl

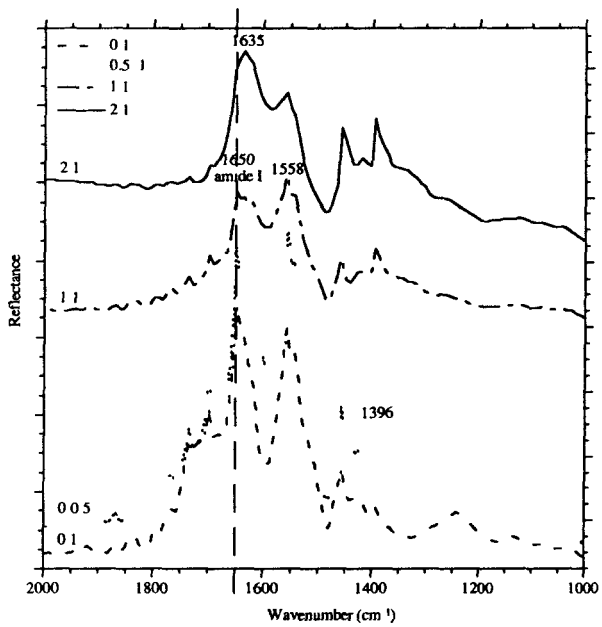

FIGURE 7 ATR-FTIR spectra of U(VI)-γ-PGA as a function of U(VI) glutamate subunit ratio, 5 mM exopolymer (based upon glutamate subunit), 0 01 M NaCl, pH 3

found the exopolymer produced at 37 °C for 12 h provided the highest M_w values and narrowest molecular weight distribution, thus, all experiments were carried out on polymer produced under these conditions The isolated polymer had an average M_w = 800 000 g mol^{-1}, which corresponds to 6200 monomeric subunits (glutamate units) The production of high molecular weight γ-PGA is in agreement with the work of others who have found that the polymer has molecular weights ranging from 300 000 g mol^{-1} to 4 1 million g mol^{-1} (*24, 27, 42*)

Unpurified polymer was rapidly degraded, suggesting that active depolymerase might be present in the cell free supernatant or that some cells (containing depolymerase) were not adequately removed during the initial separation process Because the polymer degradation was limited, it suggests that the latter occurred There are conflicting reports

about where, in relation to the cell, the exopolymer depolymerase is localized (*28, 34, 43*), and further experimentation is needed to resolve this question Once purified, however, the exopolymer was very stable at both 4 and 15 °C, with slow degradation at room temperature, suggesting either an enzymatic or a chemical mechanism for this degradation Chemical breakdown of PGA occurred in the presence of 1 M HCl, although the polymer tolerated up to 0 1 M HCl without significant degradation

The protonation behavior of the capsule was followed qualitatively using ATR-FTIR The IR stretching of the protonated carboxylate exists clearly at pH 3 0 but disappears at pH 5 45 (Figure 7), indicating that the carboxylate group is totally deprotonated at pH 5 45 Therefore, the pK value for the α-carboxylate group of the exopolymer is greater than that for the γ-carboxylate group of glutamic acid, which is 2 23 (*44*) The increase in pK may be due to intramolecular hydrogen bonding with the amide group For comparison, the pK for the γ-carboxylate group of synthesized α-poly(L-glutamic acid) (molecular weight 68 000 g mol^{-1}) has been determined to be 4 60 in 0 1 M KCl solution at 25 °C (*45*) This value is slightly greater than that for glutamic acid, which is 4 42 (*44*)

Conformation. The conformation of macromolecules changes with temperature, solution pH, electrolyte, macromolecule concentration, and solvents (*31, 46, 47*) The three conformations for polypeptides or proteins, α-helix, β-sheet, and random coil may be distinguished spectroscopically The α-helical structure has infrared spectral bands at 1655 (amide I stretch) and 1550 (amide II stretch) cm^{-1}, while the amide I band shifts to 1630 cm^{-1} for β-sheet structures and the amide II band to 1535 cm^{-1} for random coils (*47*) Our experimental results demonstrate that the conformation of the γ-PGA exopolymer is affected by solution pH, ionic strength, and concentration At low pH, the low-concentration exopolymer (0 1% w/v) is protonated and exhibited a helical conformation, presumably due to hydrogen bonding This structure is confirmed by the two bands at 1650 cm^{-1} for amide I and 1558 cm^{-1} for amide II (Figure 4) (*47–49*) At neutral to high pH, the amide I band shifted from 1650 to 1635 cm^{-1} (Figure 6), suggesting the deprotonated form of the molecule adopts a β-sheet structure Presumably, the negatively charged carboxylate groups destabilize, and there are not protons available to maintain, the hydrogen-bonded helical structure At low concentrations (0 1% w/v) and pH > 7, γ-PGA is assumed to have an elongated, stiff confirmation (*32*) A similar structural change with pH has been substantiated with poly(α-L-glutamic acid) by measuring specific rotation at 589 nm and intrinsic viscosity along with acid–base titration (*50–52*) The conformation of the α-PGA changes from helix at low pH to random coil at high pH once approximately 40% of the carboxylate groups have ionized

Salts can affect electrostatic interactions in macromolecules by the component anions and cations interacting with accessible charged or polarized sites Because cations can replace protons in their interactions with functional groups by do not form hydrogen bonds, increasing ionic strength will weaken attractive inter- and intrachain charge–charge interactions, subsequently influencing the conformation of biopolymers At low ionic strength, the bacterially produced exopolymer has a helical conformation As ionic strength increases, the structure changes to a mixture of helix and β-sheet at 0 1 M and to predominantly β-sheet at 0 5 M (Figure 4) This is in agreement with destabilization of α-PGA by neutral salts such as NaCl, KCl, and LiBr (*31*)

As the concentration of biopolymer increases, the influence of intermolecular interactions on the structure increases relative to the influence of intramolecular interactions by which helical structure is maintained At low concentration, the bacterially produced exopolymer is in a helical conformation, while at high concentration (25 mM as glutamate), its structure becomes a β-sheet as evidenced by the two bands at 1635 and 1558 cm^{-1} (Figure 5)

Complexation of U(VI) by the exopolymer also alters its conformation from helix to β-sheet structure (Figure 7) This change does not occur until the U(VI) concentration is 10 mM, with a U(VI) glutamate ratio of 2 1 at pH 3 At this concentration of U(VI) and this ratio, the U(VI) ions might play a role in linking exopolymer molecules through covalent bonding with carboxylate groups, thus disfavoring the helical structure This reasoning is consistent with a binuclear bridging structure for the U(VI)–exopolymer complex (see also below for details) Data obtained by Inoue indicates that complexation of Cu(II), Cd(II), and Ni(II) by α-PGA failed to affect its helical structure at the monomer metal ratios of 9 24, pH 3–7, and a metal concentration of 0 5 mM (*53*), suggesting that interactions of the exopolymer with the linear dioxo uranyl (effective charge 3 3) differ from those with divalent cations

U(VI)–Glutamate and –Exopolymer Complexes Metal ions may be complexed by the carboxylate group of an organic compound in a few different ways, with the manner of complexation determining the symmetry, bond strength, and bond angle These parameters affect the IR frequencies of the normal vibrational modes of the carboxylate group Based on single-crystal studies of metal–carboxylate complexes, general diagnostic criteria have been developed to distinguish various coordination structures of metal–carboxylate complexes (*54*) By comparing the IR spectra of a metal complexed carboxylate group with the IR spectra of free carboxylate group, it is possible to infer the bonding of the metal–carboxylate complex When the frequency difference, $\Delta\nu$ (where $\Delta\nu = \nu_{asymmetric} - \nu_{symmetric}$ for the COO^- group) for the metal–carboxylate complex is lower than that for the free carboxylate, a mononuclear bidentate complex is present, i e , one carboxylate group forms two bonds with one metal ion If the value of $\Delta\nu$ for the metal–carboxylate complex is greater than that for the free carboxylate, a mononuclear monodentate complex is present, i e , only one oxygen of the carboxylate group binds with one metal ion When $\Delta\nu$ differs little between the two forms, a binuclear bidentate complex is present, i e , two oxygens of a carboxylate group each form a bond with two metal ions Application of in situ spectroscopic techniques such as ATR-FTIR makes it possible to distinguish among coordination structures of aqueous phases For example, Fourest and Volesky (*55*) used ATR-FTIR data to determine that Cd(II) was complexed with alginate in a binuclear bidentate (bridging) structure

Consider the U(VI)–glutamate system first The $\Delta\nu$ value for the free carboxylate on glutamate is 161 cm^{-1} (1558 cm^{-1} for the asymmetric and 1397 cm^{-1} for the symmetric) (Figure 3), which is larger than the value of 138 cm^{-1} (1549–1411) for the U(VI)-complexed carboxylate group This difference indicates that the carboxylate group of glutamate forms a mononuclear bidentate complex with U(VI) ions Since only two bands were observed for the U(VI) complexed carboxylate, it is reasonable to postulate that both the α- and γ carboxylate groups on glutamate form the same complex with U(VI), i e , mononuclear bidentate In a similar fashion, citrate was found to form a mononuclear bidentate U(VI) complex(*56*)

For the U(VI)–exopolymer system, the $\Delta\nu$ values have little difference between the free and U(VI)-complexed carboxylate groups (Figure 7) This suggests a binuclear bidentate complex of U(VI) with the carboxylate group on the exopolymer This binding mechanism implies that the bacterially produced exopolymer should have a U(VI) sorption capacity twice its charge density provided that all sites on the exopolymer are accessible to U(VI) ions and complex with one chain Since the β-sheet structure was formed at

the 2 1 ratio as discussed above, it is possible that each U(VI) ion functions as a bridge connecting two chains of exopolymer In this case, the U(VI) binding capacity of the exopolymer equals its negative charge density The calculated negative charge density (based upon the carboxylate groups) of the exopolymer is 7 5 mmol g^{-1} This value is comparable to the 8 2 mmol g^{-1} McLean et al (*29*) reportedly determined at a different pH using the Na^+ adsorption method We note that while in this study a rather high concentration of uranyl was needed to induce the conformational change, the uranyl-to-polymer ratio is more important for environmental consideration (The relatively high concentrations used were necessary to obtain meaningful spectra and reasonable signal/noise) At the 2 1 ratio, the conformation changed, this is a condition which may occur under environmental conditions, particularly at the leading edge of a contaminant plume

The high metal affinity and binding capacity of exopolymers have been previously reported (*17, 18, 22, 29, 57*) Chen et al (*17*) found that bacterial exopolymers present a significant ability to release Pb(II) and Cd(II) adsorbed to sediment (90% released) Since it has been shown that polymer sorption by sediment is negligible, metal complexation is likely the predominant mechanism for metal release from sediment (*17*) Polymer-complexed metal ions are likely to have a lower toxicity to organisms than free metal ions (*58*)

Many species of bacteria produce exopolymers in soils, sediments, and waters The significance of exopolymers conformational changes are potential effects on effective surface charge, which is responsible for metal binding The α-helical structure is likely maintained via hydrogen bonding along the helix between the α-carboxylate and the backbone carbonyl groups This conformation places some of the carboxylate group inside the helix and inaccessible for metal binding The β-sheet structure may be formed through hydrogen bonding between the amino and carbonyl groups on the backbone, leaving the α-carboxylate group free Thus, the β-sheet conformation should exhibit the maximum negative surface charge density and largest metal-binding capacity Further studies are needed to examine how the change in confirmation alters the effective surface charge density of the exopolymer In terms of application to an environmental site, it is more complicated considering the variability of conditions, such as soil pH, contaminant type and concentration, and exopolymer concentration For most soils with pH ranging from 5 to 6 5, if metal contaminants are present, exopolymers should bind them and adopt the β-sheet conformation Consequently, exopolymers in the environment are able to bind radionuclides at a relatively high level and may considerably affect their fate and transport

Bacterially produced, unpurified exopolymers degrade rapidly, suggesting they may be unimportant in environmental migration and potential bioremediation However, the biodegradation of exopolmers present in contaminated soils, sediments, and waters may differ from what has been observed in relatively pure experimental systems The degradation of organic compounds has been shown to be strongly inhibited by sorption to soil surfaces or by binding to metals For example, Francis et al found that metal–citrate complexes, in contrast to free citrate, were resistant to degradation and that the resistance resulted from the chemical nature of the complexes, not from the toxicity of the metals (*59*) Czajka demonstrated that the presence of Pb(II) has a significant effect on the degradation of bacterially produced exopolymers in both sediment column and batch experiments They concluded that Pb(II) present in soils may inhibit the degradation of exopolymers (*18*)

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