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Biosorption of Uranium and Copper by Biocers

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Biological ceramic composites (biocers) made according to aqueous sol−gel protocol were used as selective metal binding filters. The biological component of the biocers *Bacillus sphaericus* JG-A12 was isolated from a uranium mining waste pile. Vegetative cells and spores of this strain are known to bind selectively U, Cu, Al, Cd, and Pb in large amounts. Sol−gel ceramics were prepared by dispersing vegetative cells, spores, and stabilized surface-layer proteins (S-layer) in aqueous silica nanosols, gelling, and drying. The biosorption of uranium and copper by the three kinds of biocers and by their single components was investigated with dependence on time, concentration, and preparation conditions. Biocers with cells possess the highest binding capacity compared to matrixes with spores and an S-layer. Freeze-drying of prepared biocers or adding water-soluble compounds as sorbitol lead to higher porosity and faster metal binding. Uranium was bound mainly to the biological component but also to the SiO_2 network. In contrast, copper was only bound by the cells, spores, or S-layer. Bound uranium and copper were completely removed by washing with aqueous citric acid.

Introduction

As a current trend in the field of bio-engineered materials, sol−gel technology opens up new vistas in immobilization of biocomponents. The favorable characteristics of inorganic oxide matrixes, for example, (a) good mechanical, thermal and photochemical stability, (b) high spectral transparency as far as the deep UV region, (c) not a food source for microorganisms since they are toxicologically and biologically inert, and (d) controlled matrix porosity, offer important advantages when combined with biocomponents. Adjustability of the matrix porosity is important for the degree of immobilization of biocomponents and efficient diffusion processes and reactions. Depending on experimental conditions, sol−gel protocols enable encapsulation of biomolecules retaining their conformation and catalytic activity. Even viable cells such as yeasts[1−5] or bacteria[6−11] can be embedded and still maintain their viability ("living composites, biocers").[12] The reliable immobilization of biocomponents is crucial for application of biocers in remediation technologies.

This paper deals with the characteristics of sol−gel immobilized cells, spores, and purified surface-layer protein of *Bacillus sphaericus* JG-A12 used as metal selective filter materials. *B. sphaericus* JG-A12 was isolated from the uranium mining waste pile "Haberland" near the town of Johanngeorgenstadt, Saxony, Germany. Vegetative cells and spores of this strain accumulate selectively large amounts of U, Cu, Pb, Al, and Cd from the highly polluted drainwaters of this uranium mining waste.[13] This strain possesses a square protein lattice (S-layer). As an outermost component of the cell wall, the S-layer may function as a molecular sieve and ion trap.[14−16] It was demonstrated that the isolated S-layer lattices interact with several metals by forming nanoclusters.[17−20] The ability of *B. sphaericus*

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to interact with heavy metals and its geographic origin make it a good candidate for preparation of bacteria-based ceramics (biocers) for in situ bioremediation of uranium mining waste pile waters. The goal of the work was to retain the high biosorption capacity known for native *B. sphaericus* by preparation of biocers under mild conditions using an aqueous sol−gel protocol. Variations in porosity and structure of the biocers were obtained by using alternative drying procedures. Sorption and desorption of uranium and copper by these biocomposites were investigated and visualized by energy-dispersive X-ray analysis (EDX).

Materials and Methods

Preparation of the Aqueous Silica Nanosol. Silica sols were prepared by stirring 10 mL of tetraethyl orthosilicate (TEOS), 40 mL of ethanol, and 20 mL of 0.01 N HCl catalyst for 20 h at room temperature. For aqueous silica sols the ethanol was evaporated by leading air through the solution. The evaporated ethanol was substituted by water. The resulting mean silica particle size in the aqueous nanosol was 6 nm (by Zetasizer 1000HS/Malvern).

Bacterial Growth. *B. sphaericus* JG-A12 was grown to the mid-exponential growth phase in 30 mL of nutrient broth (8 g L^{-1}, Difco), pH 7.0, in 100-mL flasks which were shaken at 30 °C. The bacterial suspension was used to inoculate 6 L of nutrient broth in 7.5-L bioreactors (Ochs, Bovenden/Lenglern). All fermentations were performed as batch cultures at 30 °C, a stirring speed of 500 rpm using a magnetic stirrer, and flow rates of 3 L of air/min. Bacterial growth was followed by measuring optical densities at 600 nm using a Pharmacia Biotech spectrometer Ultrospec 1000. Cells were harvested at the late exponential growth phase by centrifugation at 10 000*g* for 20 min.

Preparation of Spores. For sporulation the nutrient broth medium was supplemented with 10 mg L^{-1} $MnSO_4 \cdot H_2O$ and fermentation was carried out until cells were completely sporulated. Spores were harvested by centrifugation at 10 000*g* for 20 min and washed two times with ultrapure water at 4 °C. Resuspended spores were treated alternately with 0.2 mg L^{-1} lysozyme (Sigma-Aldrich Chemical Co., Deisenhofen) and 0.1 mg L^{-1} trypsin (Boehringer, Ingelheim) at 4 °C for the complete lysis of intact cells and cell wall fragments, washed twice, and stored in ultrapure water at 4 °C.

Preparation of Cell Wall Fragments and Isolation of S-layer Protein. Intact cells were washed once, centrifuged, and resuspended using the same standard buffer solution (50 mM Tris−HCl, 1 mM $MgCl_2 \cdot 6H_2O$, 3 mM NaN_3, pH 7.5). For removing bacterial flagella, the suspension was homogenized in a rotating-blade bender IKA T8 (IKA Labortechnik, Stauffen) at maximum speed for 10 min on ice. Flagella free cells were harvested by centrifugation at 6000*g* for 10 min at 4 °C, resuspended 1:1 in standard buffer, and mixed with a few crystals of DNAse II and RNAse. The cells were disintegrated in a mixer mill at 4 °C with glass beads with a diameter of 0.1 mm. After removal of the glass beads and unbroken cells by differential centrifugation, cell wall fragments were suspended in standard buffer. Plasma membranes were solubilized in 1% Triton X-100 in the buffer solution for 10 min at RT, and the remaining cell wall fragments were washed twice. Peptidoglycan was lysed by incubating the samples in a buffer solution containing 0.2 mg mL^{-1} lysozyme for 6 h at 30 °C. The S-layer fraction was washed several times, resuspended in standard buffer, and stored at 4 °C.

Stabilization of the S-layer. For stabilization of the square lattice structure of the isolated native S-layer sheets they were incubated with 1-ethyl-3-(*N*,*N*′-dimethylaminopropyl)carbodiimide[21] (EDC, Sigma-Aldrich Chemie GmbH, Taufkirchen) at a concentration of 30 mg mL^{-1} in standard buffer for 48 h at 20 °C. Cross-linked S-layer was harvested by centrifugation at 12 400*g* for 30 min at 4 °C, washed three times with distilled water, and stored at 4 °C. The pH stability of the S-layer sheets was investigated by their incubation for 10 min to 48 h at RT in a buffer solution (0.1 M citric acid, 0.2 M Na_2HPO_4) with a pH from 2 to 9 and UV−vis spectroscopical analysis. Samples were scanned from 200 to 700 nm in a Ultrospec 1000 spectrometer (Pharmacia Biotech, Cambridge).

Preparation of the Biocers. Forty milliliters of aqueous silica sol was mixed with a concentrated suspension of the biocomponent. Before mixing, the pH of the aqueous silica sol was increased up to about pH 7 by the addition of NaOH. The proportion of biocomponents in the sol was as follows: 9.35 mg mL^{-1}, respectively 6.5×10^9 *B. sphaericus* cells; 3.96 mg mL^{-1}, respectively 6.5×10^9 spores; or 9.57 mg mL^{-1} S-layer. Sorbitol (20% w/w SiO_2) was added to some silica sols to achieve a higher porosity. The Biosol was poured into dishes to a layer thickness of approximately 7 mm. Gelling occurs a short time after the neutralization and the addition of the biocomponents. The gels were aged for 3 days at 4 °C, cut into small pieces, and dried at room temperature or by freeze-drying. The dry gels were sieved to particles with a size of 355−500 μm.

Biosorption of Heavy Metals. Investigations were carried out with 200 mg dry weight of sieved silica gel or biocer particles containing 36.4 mg of bacterial biomass corresponding to 2.6×10^{10} *B. sphaericus* cells, 17.23 mg of spore biomass corresponding to 2.8×10^{10} spores, or 36.4 mg of S-layers. The metal binding capacity of the same amounts of free cells, spores, and S-layers was measured as well. All components were shaken in 35 mL of 0.9% $NaClO_4$, pH 4.5, with 9×10^{-4} M $UO_2(NO_3)_2 \cdot 6H_2O$ (Fluka, Deisenhofen) or $CuCl_2 \cdot 2H_2O$ (Merck, Darmstadt) at 30 °C for 48 h. The metals amount in the treated solutions was determined by inductively coupled plasma mass spectroscopy (ICP-MS) using an ELAN-5000 ICP-MS (Perkin Elmer, Wellesley). Desorption experiments were carried out after washing 2-fold with 0.9% $NaClO_4$, pH 4.5, in 35 mL of 0.5 M citric acid, trisodium salt, pH 4.5, at 30 °C for 24 h. The error range for the uranium binding experiments is 4−9% and for the copper binding experiments 4−30% (measured as duplicate or triplicate).

Electron Microscopy. Samples were embedded in liquid colloidal silver on conductive carbon sheets. After shadow casting with carbon (Baltec MED 010, BAL-TEC AG, Liechtenstein), the examinations of the biocers were performed using a Gemini 982 scanning electron microscope (LEO, Oberkochen) with an energy-dispersive X-ray analyzer (NORAN X-ray detector) at 1−5 kV.

Scanning Force Microscopy. Scanning force microscopy (SFM) investigations were carried out with a Nanoscope IIIa (Digital Instruments, Santa Barbara) in tapping mode in air. Samples were prepared by placing a droplet of the sample suspension on a Si wafer and removing excess solution using filter paper after 1 min. Then the sample was rinsed with water and air-dried.

Results and Discussion

pH Stability of the Biological Components. To use biocomponents in bioremediation processes for cleaning radionuclide and heavy metal contaminated drainwaters of different environments, the pH stability of the biological components is of special importance. While the cells and the spores of *B. sphaericus* JG-A12 are stable in acidic drainwater,[13] native S-layer dissociates at pH 4 and below (Figure 1 A). UV−vis spectra of intact protein lattices show light scattering and additionally for the protein monomer typical absorption

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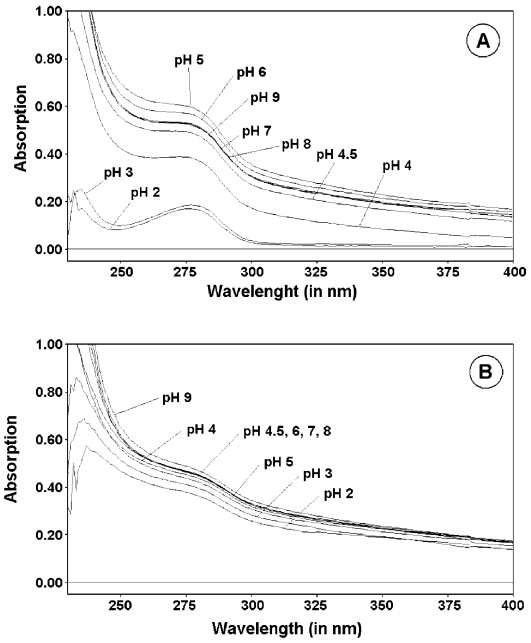

Figure 1. UV−vis spectra of *B. sphaericus* JG-A12, native (A) and EDC-cross-linked (B) S-layer sheets dependent on different pH values.

at 280 nm. Dissociated protein monomers possess only the absorption at 280 nm. The spectra presented in Figure 1A demonstrate the stability of native S-layer sheets from pH 4.5 to pH 9. At pH below 4.5 the lattice dissociates in monomers. When solutions were neutralized again, the S-layer monomers did not recrystallize to lattices. To increase their stability, the S-layers had to be cross-linked. Common cross-linking reagents for proteins are glutaraldehyde or 1-ethyl-3-(*N*,*N*′-dimethylaminopropyl)carbodiimide. Glutaraldehyde treatment of proteins is less suitable because in this case serious modification of the protein occurs. Infrared spectroscopic analysis demonstrates an influence of the permanent linkage of glutaraldehyde polymers to the protein on the metal binding capability.[22] In contrast, native and EDC stabilized S-layers bound metals to the same functional groups. EDC treated S-layer did not dissociate at pH values from 2 to 9 (Figure 1B). Scanning force microscopical investigation of the stabilized S-layer showed the original lattice parameters, but several protein lattices were linked together.

Immobilization of the Biocomponents. To use biocomponents as effective parts of the filter materials in the bioremediation processes, their reliable immobilization has to be achieved. Only under these prerequisites repeated usage of the biocers for metal binding is possible and the mobilization of the cells, spores, and S-layer can be prevented.

Besides immobilization, the porosity and thus the accessibility of the biocomponent within the matrix are of importance. Air drying of the gel matrix results in a noticeable shrinkage of the silica network, which accompanies the increasing strength of the matrix. Addition of highly soluble compounds such as sorbitol to the silica sol yields a more porous structure and a lower shrinkage of the silica network. After drying, the pores are formed by leaching of sorbitol from the silica matrix

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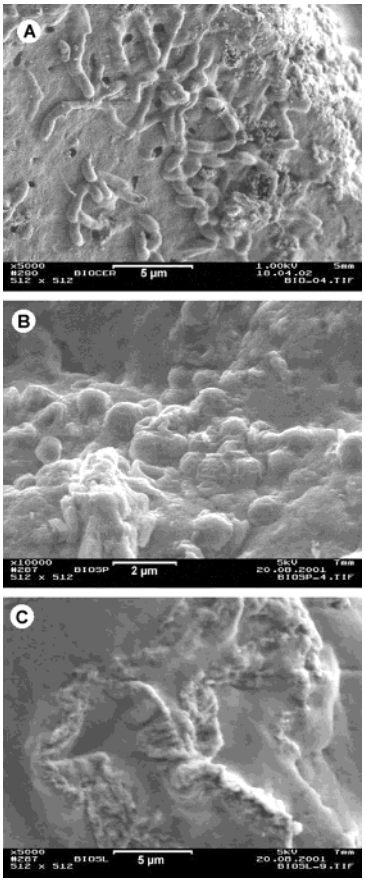

Figure 2. Scanning electron micrographs of immobilized *B. sphaericus* JG-A12: (A) cells, (B) spores, and (C) S-layer protein in sol−gel ceramics.

during incubation in aqueous solutions. The more porous structure of the biocers is seen by REM. Recently, Wei et al.[23] proved that a linear correlation exists between the net BET surface area, pore volume, and glucose content (like sorbitol) prior to water extraction of a sol−gel silica matrix. Another possibility for obtaining a highly porous structure is freeze-drying of the silica gel. In this case a small volume reduction takes place which is accompanied by a low stability of the silica network. The structures of different biocers were investigated by scanning electron microscopy (Figure 2).

While free and immobilized spores of *B. sphaericus* JG-A12 keep their dimensions, immobilized cells were smaller in size due to shrinking processes. For the same reason the S-layer structure changed from a flat protein lattice as a free component to a corrugated layer in the SiO_2 matrix. The structure of the biocer is directly influenced by the kind of the embedded biocomponent. After several washing steps, cells, spores, and S-layer inside and on the surface stayed completely immobilized. Besides a homogeneous allocation of the cells, spores and S-layer pores in the SiO_2 matrix were clearly visible. The amount of pores and channels in the biocer particle is directly connected with the inner surface and this influences the binding capacity and binding kinetics.

Metal Binding of the SiO_2 Matrix, the Free *B. sphaericus* Cells, Spores, and S-layer. As known

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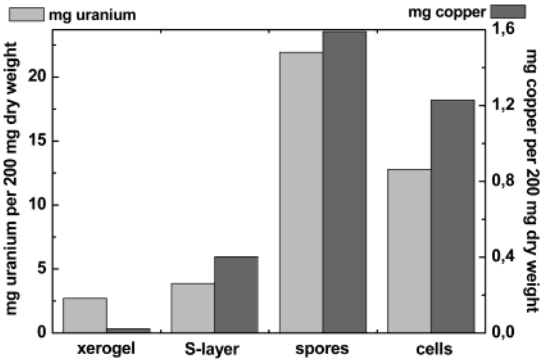

Figure 3. Metal binding by 200 mg dry weight of xerogel, *B. sphaericus* JG-A12 S-layer, spores, and cells.

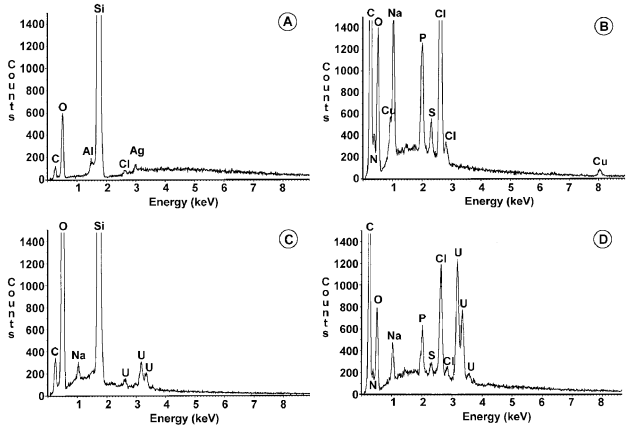

Figure 4. EDX spectra of xerogel samples incubated with copper (A) and uranium (C) and of *B. sphaericus* cells incubated with copper (B) and uranium (D).

from the investigations of free and immobilized yeast cells, the binding of diverse metals is different.[5] For this reason the metal binding of the silicate matrix, free cells, spores, and S-layer was investigated before entrapment. The uranium and copper binding were measured after a single incubation of 200 mg of xerogel, 36.4 mg of cells, 17.23 mg of spores, and 36.4 mg of S-layer with 35 mL of 9×10^{-4} M $UO_2(NO_3)_2 \cdot 6H_2O$ or $CuCl_2 \cdot 2H_2O$ each in 0.9% $NaClO_4$, pH 4.5, for 48 h. The amounts are equal to 7.5 mg of uranium or 2.0 mg of copper, respectively. The results were normalized to 200 mg dry weight (Figure 3). Spores possess the highest binding capacity for uranium and copper. Under the selected conditions spores bind 21.9 mg of uranium and 1.6 mg of copper per 200 mg dry weight followed by intact cells which bind 12.8 mg of uranium and 1.2 mg of copper, S-layer with 3.9 mg of uranium, and 0.4 mg of copper and last the xerogel with 2.7 mg of uranium and 0.02 mg of copper. Binding capacities were standardized to the dry weight. In the case of the cells and the spores same dry weights mean different number of particles; 200 mg dry weight of cells is equal to 1.4×10^{11} cells or 3.3×10^{11} spores. Against this background, cells possess higher binding capacities for both metals. All components bind at least 10-fold more uranium compared to copper. Additionally, copper was only bound to cells, spores, and S-layer, but not to the xerogel (Figure 4). Involved in metal binding are NH−,[24] COOH−, or PO_4− groups.[25]

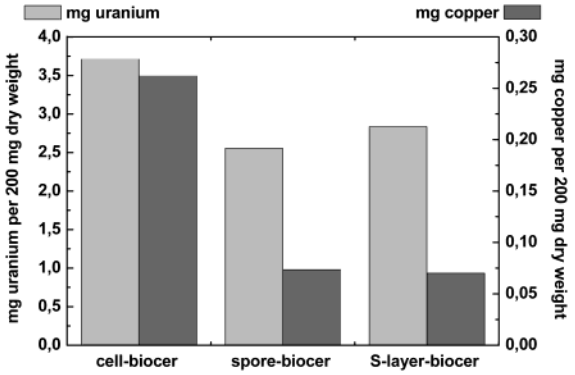

Figure 5. Metal binding of 200 mg dry weight of biocers with embedded *B. sphaericus* JG-A12 cells, spores, and S-layers.

In the case of *B. sphaericus* JG-A12 cells uranium is mainly bound by phosphate groups[26,27] on the surface of the cells. Copper complexation occurs at either extracellular polymers or parts of the bacterial cell walls. As spores possess a higher density than cells and a lower water content, metals were also bound only on the surface, where similar functional groups are present.

At last the biosorption of uranium and copper on S-layer sheets is again a superficial interaction between the present NH−, CO−, COOH−, and PO_4− groups and the metals. In all cases the metals were bound to the surface of the biocomponents,which makes desorption easy. These properties make all mentioned biomaterials suitable for the preparation of a filter material for repeated biosorption.

Metal Binding of the Biocers. For the preparation of 200 mg dry weight of biological ceramics, 36.4 mg of cells, 17.23 mg of spores, and 36.4 mg of S-layer protein were used. Cell biocers bind 3.7 mg of uranium and 0.26 mg of copper, spore biocers 2.6 mg of uranium and 0.07 mg of copper, and S-layer biocers 2.8 mg of uranium and 0.07 mg of copper per 200 mg of biocer dry weight (Figure 5). The composite material with cells shows in both cases highest binding capacities followed by the S-layer-ceramic and the spore-ceramic. Only the latter shows significant lower binding capacities as estimated from the amounts of bound metal by the xerogel and by the spores. This means that for uranium 58% and for copper 47% of the theoretical values were reached.

In contrast the measured binding capacity of the biocer with cells and S-layer reached 81% or 97% for uranium and 108% or 74% for copper compared to their theoretical binding capacity. Possibly this can be ascribed to drying effects of the biocers. The water content of cells and S-layer is high; in contrast, it is very low for spores. During the drying step cells, S-layers and the xerogel matrix are shrinking while spores keep their original structure. This leads in the case of spore biocers to a densification of the interface between the gel matrix and the spores and at last to a decrease of the access to the binding sites.

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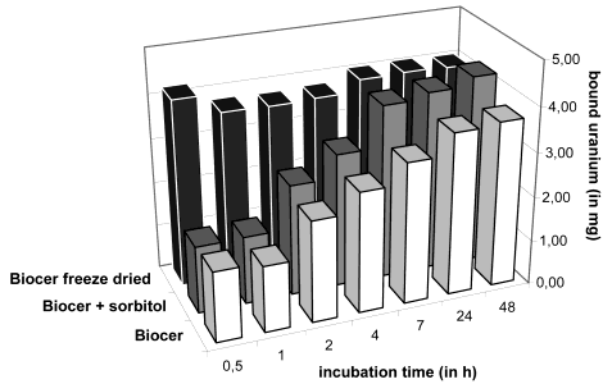

Figure 6. Uranium binding of different porous biocers with embedded *B. sphaericus* cells.

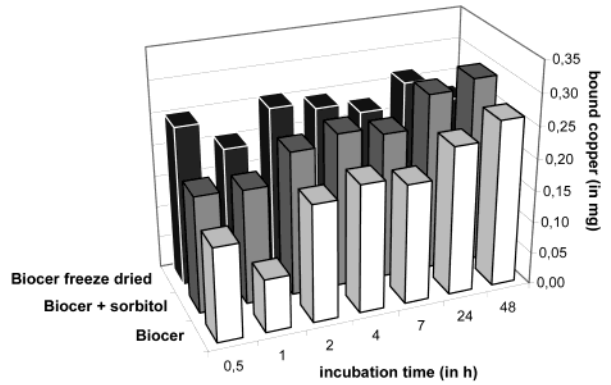

Figure 7. Copper binding by different porous biocers with embedded *B. sphaericus* cells.

Metal Sorption of Biocers with Higher Porosity. Freeze-drying of gels or the addition of water-soluble compounds such as sorbitol to the sol lead to a more porous structure of the material. Figure 6 and Figure 7 show the binding kinetics of uranium and copper for air-dried biocers, air-dried biocers produced with the addition of sorbitol, and freeze-dried biocers.

As evident from the figures, the influence of the material structure of the biocers on the process of metal binding is significant. Freeze-dried biocers bind the maximum amount of uranium or copper already at the first measurement after half an hour. In addition, two more porous biocers bind higher amounts of uranium. For copper such a trend is not so pronounced. Only biocers produced by the addition of sorbitol show a higher binding capacity.

Reusing of the Biocers. For a cost-efficient renewed usage of the biocers as filter material the mechanical strength and the easy removal of the bound metal is crucial. Both properties and also a high metal binding capacity are achieved by production of the air-dried biocers. Freeze-dried biocers show a faster metal binding but the lower stability of the matrix makes them less suitable for technical application. Desorption experiments were carried out with 0.5 M citric acid, trisodium salt, pH 4.5, at 30 °C. For this the biocers were washed first two times in 0.9% $NaClO_4$, pH 4.5, followed by a 2-fold incubation in 0.5 M citric acid for 24 h. For both metals a complete removal of the bound metal from the biocer was achieved. Differences exist in the bond strength of uranium or copper at the biocers. Uranium was removed from the biocers to 10−19% in the first two washing steps. The following incubation in 0.5 M citric acid resulted in a desorption of 78−87%. Reincubation in aqueous citric acid removed the remaining uranium bound to the biocers. In contrast, copper was removed more easily from the biocers; 55−65% of copper was removed by the first two washing steps. The following incubation in citric acid results in a desorption of an additional 33−38%, and 3−7% by the next incubation.

Conclusions

Binding experiments with intact cells, spores, and EDC stabilized S-layer protein of *B. sphaericus* JG-A12 demonstrate high binding capacity for uranium and for copper. The pure silicate matrix in contrast binds less uranium and no copper. Using an aqueous sol−gel process for embedding the mentioned biocomponents in silica gels, it is possible to construct a filter matrix with a homogeneous structure and completely immobilized biocomponents. Moreover, this process did not influence the metal binding capability of cells and S-layers. Spore containing biocers, however, possess significantly lower binding capacities in comparison to the free components. We suggest that this is connected to the formation of a more compact bioceramic with lower porosity due to the small size and rather high density of the spores, which are nearly water free. The metal binding capacity and the kinetics of the process are positively influenced by adding water-soluble compounds such as sorbitol or by freeze-drying because of higher porosity achieved by this treatment. It is important to stress that both metals can be completely removed from the free biocomponent and from the biocer by using aqueous citric acid. Due to the high stability of air-dried biocers, the safe immobilization of the embedded biocomponents, the high metal binding capacity, and the simple and complete removal of the bound metals, the described biocers are suitable for reversible usage without influencing the binding capacity (personal communications Dr. H. Quast, Kallies Feinchemie AG, Sebnitz).

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