Anode potential-dependent protection of electroactive biofilms against metal ion shock via regulating extracellular polymeric substances

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Abstract
Extracellular polymeric substances (EPS) have been considered as a barrier for toxic species penetration into the cells, but their function in protecting electroactive biofilms (EABs) had been rarely revealed. In this study, the anode potential was used to regulate the EPS quantity and components in mixed-culture EABs, where their resistance to Ag⁺ shock was assessed. The results showed that the EAB grown at 0 V showed the highest anti-shock capability by the Ag⁺ exposure compared to those grown at −0.2, 0.2, and 0.4 V. The EAB produced at 0 V had both of the highest amounts of loosely bound EPS (LB-EPS; 61.9 mg-EPS/g-VSS) and tightly bound EPS (TB-EPS; 74.8 mg-EPS/g-VSS) than those grown under other potentials, where proteins and humic acid were the predominated components. The abundance of genes associated with EPS biosynthesis were also conﬁrmed to be related with the applied anode potentials, based on the metagenomic analysis. Considering proteins and humic acid in LB-EPS showed positive linearity with the current recovery and viability of the EABs, these two main components might play important roles in reducing the Ag⁺ toxicity. Synchronous fourier transform infrared (FTIR) spectroscopy integrated two-dimensional correlation spectroscopy (2D-COS) analyses further conﬁrmed that the oxygen and nitrogen moieties (i.e. amide, carbonyl C=O, phenolic, and C—O—C) in proteins and humic acid of the LB-EPS were response for the binding with the Ag⁺ to prevent the penetration into the cells. The underlying molecular mechanisms of EPS in protecting EABs from the Ag⁺ shock explored in this study can provide implications for developing new methods to construct highly stable EABs.

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1. Introduction

As the most important part of bioelectrochemical system (BES), electrochemically active biofilms (EABs) can directly exchange electrons with extracellular solid carriers (i.e., electrodes) to generate electricity (Borole et al., 2011; Sevda et al., 2018). However, recent researches have shown that EABs are vulnerable to external disturbances, especially heavy metals shock, antibiotic exposure, and pH changes (Logan and Regan, 2006; Lovley, 2012). The electron pathways and mass transfer inside EABs can be greatly changed by external disturbances (Chen et al., 2015a; Men et al., 2015; Patil et al., 2011), which consequently inhibits the bioelectrocatalytic activity of BESs (Li et al., 2016; Logan, 2009; Logan et al., 2006). Therefore, strengthening the stability of EABs has become an issue of great concern for practical applications of BESs.

Extracellular polymeric substances (EPS) mainly consist of polysaccharides, proteins and lipids (Flemming et al., 2016); and have the ability to enhance bacterial adhesion, promote biofilm structural development, provide a protective barrier and store nutrients for biofilm growth (Adav et al., 2008; Vu et al., 2009; Xue et al., 2012). Recent studies have confirmed that EPS play important roles in protecting biofilms against external stress (Li et al., 2019; Wang et al., 2013; Xue et al., 2012). EPS can effectively bind heavy
metal cations (such as Ag⁺, Pb²⁺ and Zn²⁺) and act as a permeability barrier to hinder intracellular penetration of metal ions (Nocelli et al., 2016; von Canstein et al., 2008). In addition, EPS have been reported to significantly increase the resistance of biofilms to antibiotics and disinfectants through mechanisms including the creation of complex structures, limitation of the transportation of xenobiotics and retention of the organic matter in the biofilms (Xue et al., 2012). The groups (e.g., amino, carboxyl, and phenol) in EPS have been considered to be the active sites for complexing various toxicants to increase the resistance of biofilms (Duan et al., 2013; Zhu et al., 2012). These results provide valuable insights regarding the protection of EABs against external stresses, such as heavy metals and antibiotics, by stimulating EPS formation.

Recent studies have shown that controlling the operation parameters of BESs can regulate the formation and structure of EABs on electrode surface to enhance their activities. In recent studies, electrode potentials have been confirmed to be significant in regulating biofilm formation and optimizing BES performance (Patil et al., 2011; Peng et al., 2016). Carmona-Martínez and Feng et al. found that the biofilm biomass and current density in Shewanella putrefaciens inoculated BES increased with elevated anode potential (Carmona-Martínez et al., 2013). Li et al. (2017) proved that Geobacter sulfurreducens grown at −0.2 V vs SHE had the highest cytochrome C contents and reducing capacities among biofilms grown under various conditions. Peng et al. (2016) found that exocellular electron transfer pathways, rather than biofilm community variation, were responsible for different EAB activities at various set potentials. In addition to the electrochemical activity of the biofilms, the production and structure of EPS were highly affected by the applied anode potential in pure-culture Geobacter biofilms (Yang et al., 2019a). However, the variations in EPS composition in mixed-culture EABs at different anode potentials have not yet been clearly clarified. Moreover, the role of EPS in EABs for protecting biofilms from anti-shock effects has remained largely unexplored.

Therefore, the aim of this study is to investigate EPS variations in mixed-culture EABs formed at different potentials and evaluate their effects on the stability of EABs against metal ion shock. EABs were constructed at four different anode potentials (i.e., −0.2, 0, 0.2, and 0.4 V vs SHE), where the variations in EPS in the as-formed biofilms were examined. Ag⁺, one of the most toxic metal ions for bacteria (Silver et al., 2006), was chosen as the external stimulation to explore the anti-shock properties of the EABs as the biofilms became mature. The relationship between the EPS composition and the tolerance of EABs was established to elucidate the key components of EPS in protecting EABs against Ag⁺ shock. To the best of our knowledge, this is the first study to link the electrode potential, EPS composition and the resistance to external stimulation for a mixed-culture EAB. The results may be useful for establishing highly stable EABs against external stresses and broadening their application in the BES platform for environmental remediation.

2. Materials and methods

2.1. Electroactive biofilm growth and Ag⁺ exposure

The EABs on the electrodes were developed using a single chamber BES. The BES was constructed using 100 mL cylindrical glass container and a polytetrafluoroethylene lid, where graphite plates (1.0 × 2.0 cm) and titanium wire were used as the working and counter electrodes, respectively, and a saturated calomel electrode (SCE) was used as the reference electrode. The reactors were run in duplicates at 30 °C, which were inoculated with 20 mL effluent of a previous well operating BES (originally acclimated from anaerobic sludge; Yuan et al. (2011)). A multichannel potentiosat (CHI 1000C, CH Instrument, Shanghai, China) was used to set four different anode potentials (−0.2, 0, 0.2 and 0.4 V vs SHE) to enrich EABs and record the current generations of the BESs. The culture medium contained NaAc (1 g/L), NaH2PO4·2H2O (2.84 g/L), Na2HPO4·12H2O (11.4 g/L), NH4Cl (0.31 g/L), KCl (0.13 g/L), vitamin solution (10 mL/L), and mineral solution (10 mL/L) (pH = 6.8).

The Ag⁺ solution was prepared by dissolving AgNO₃ in deionized water. After the mature EABs were achieved, the anodes were taken out and inserted into the 60 mg/L Ag⁺ solution in duration of exposure (1, 2 and 3 h). The anodes were then washed using distilled water to remove Ag⁺ solution and re-installed in the BESs for electrochemical tests. The EABs were carefully scratched off the electrodes with a cell scraper for the EPS extraction, quantification and characterization.

2.2. EAB characterization

Chronoamperometry was used to record the current generation of the BESs with a multichannel potentiosat. Turnover cyclic voltammetry (CV) of the EABs was conducted by scanning potentials in a range of −0.8 to 0.2 V vs SHE with a scan rate of 1 mV/s (Feng et al., 2016). A modified Bradford protein assay kit (C503041, Sangon Biotech Co., Ltd., Shanghai, China) was used to determine the biofilm biomass. The morphologies of the biofilms were characterized using scanning electron microscopy (SEM) (Yuan et al., 2013). The volatile suspended solids (VSS) of the EABs were measured according to a standard method (Ye et al., 2011). The viability of the cells in the biofilms was evaluated by confocal laser scanning microscopy (CLSM, Biorad, Radiance 2100 MP, USA). Biofilms before and after being exposed to Ag⁺ were observed by scanning electron microscopy and energy dispersive X-ray spectroscopy (SEM-EDS; FEI, Talos F200S, Czech); the biofilms were also stained with a LIVE/DEAD BacLight Bacterial Viability Kit (L7007, Thermo Fisher Scientific Inc., USA), rinsed in sterile PBS, and examined by CLSM (Wang et al., 2016). The three-dimensional biofilm structure was reconstructed and analyzed using ZEN (Blue Edition, Carl Zeiss). The percentage of living cells was calculated and analyzed by using Comstat 2.1 software (Xue et al., 2012).

2.3. High-throughput sequencing and metagenomics analysis of EABs

Samples from the biofilm layers at the working electrode were gently scraped from the biofilm carriers under sterile conditions. After centrifugation, microbial DNA was extracted from the residual biomass using an EZNA™ Mag-Bind Soil DNA Kit (OMEGA, GA, USA) according to the manufacturer's instructions. The V3–V4 region of the bacterial 16S rRNA gene was amplified, and the purified amplicons were analyzed on an Illumina MiSeq platform (Illumina, San Diego, USA) by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). After screening, the sequence data for each operational taxonomic unit (OUT) were assigned using USEARCH v5.2.236 with 97% sequence identity. The results were classified at the phylum and genus levels.

In addition, to better understand the potential functional contributions of the observed microbes, the metagenomic information for the biofilm was investigated according to Yin et al. (2018). The extracted DNA samples were analyzed by using the illumina MiSeq platform, and the sequences assembled by the SOAPdenovo program were then predicted by the program MetaGene (Noguchi et al., 2006). The evolutionary Genealogy of Genes Non-supervised Orthologous Groups (EggNOG) database and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database were used for functional genes annotation by searching the keywords.
adhesion protein”, “exopolysaccharide”, “alginate”, “poly-γ-glutamate”, “TCA cycle”, “ATP production”, “extracellular electron transfer related gene”, “cytochrome C”, and “pili protein”, etc. Raw metagenomic datasets were deposited into the NCBI Sequence Read Archive (BioSample accession nos. SAMN07840101 and SAMN07840102).

2.4. EPS extraction, quantification, and characterization

The EPS from the original EABs and the Ag⁺-treated EABs were extracted following a method described previously (Cao et al., 2011; Yang et al., 2019b). For EPS extraction, 3 parallel graphite plates were merged into one EAB sample, and EPS were extracted in triplicate. In brief, the EABs were first resuspended in 5 mL of NaCl solution (0.9%), and the suspension was further diluted with a solution that had been preheated to 70 °C to maintain a temperature of 35 °C in the final solution. The resulting suspension was then sheared by a vortex mixer for 2 min, followed by centrifugation at 5000 g for 15 min. The supernatant was filtered through a 0.22 μm membrane filter. The organic matter in the supernatant was collected as the loosely bound EPS (LB-EPS) (Cao et al., 2011). The tightly bound EPS (TB-EPS) in the suspension was twice washed using 0.9% NaCl solution. The suspension was resuspended in 5 mL of 0.9% NaCl solution. The suspension was then sonicated for 10 min, centrifuged at 5000 g for 20 min and washed twice using 0.9% NaCl solution. The tightly bound EPS solutions were further extracted through a 0.22 μm membrane filter. The organic matter in the supernatant was collected as the tightly bound EPS (TB-EPS) in the suspension was finally collected after filtering through a 0.22 μm membrane filter (Yang et al., 2019b). Polysaccharides in the collected EPS were determined by the anthrone method (Larsson and Tornqvist, 1996). The protein and humic acid contents were measured by a BCA Protein Assay Kit (Thermo Scientific Pierce) and a modified Lowry method (Frolund et al., 1995), respectively. DNA was measured by the diphenylamine colorimetric method (Burton, 1956). The concentration of the EPS components was expressed as a function of the VSS of the biofilms (mg/g VSS) (Fig. S1).

Then, the structure of LB-EPS was analyzed using fluorescence EEM (excitation-emission matrix) using a luminescence spectrometer (FLS1000, Edinburgh Co., UK) (Shou et al., 2018). The spectra were collected from 300 to 550 nm at 0.5 nm increments by varying the excitation wavelength from 200 to 400 nm at 10 nm increments. CLSM was also applied to investigate the distribution of EPS components in the biofilms. The detailed information for the staining procedures is described in the Supporting Information (SI-1).

2.5. Two-dimensional correlation spectroscopy (2DCOS) fluorescence and FTIR analyses

2DCOS fluorescence and FTIR analyses were performed to collect fluorescence and FTIR spectra of LB-EPS with added Ag⁺ concentrations in the range of 0–20 mg/L as an external perturbation, according to a previous study (Chen et al., 2015b). Briefly, a mixture of LB-EPS and Ag⁺ was adjusted to pH 7.0 and shaken for 12 h at 25 °C to ensure complete reaction equilibrium before conducting fluorescence and FTIR measurements. Approximately 5 mL of each solution was amended with 0.04 M KNO₃ to maintain background ionic strength, and then analyzed in three scans by synchronous fluorescence spectroscopy (Edinburgh, FSS, UK). The excitation and emission slits were both adjusted to 10 nm, the excitation wavelength range was set as 250–550 nm in 0.5 nm increments, and a scan rate of 100 cm/min was used. The remaining EPS solutions were firstly freeze-dried, and then mixed with KBr (IR grade) using an agate grinder. The mixture was compressed and subjected to the FTIR measurements by a spectrometer 8900 IR spectrometer (Nicolet 6700, Thermo-Fisher, USA). The scanning conditions were as follows: a spectral range of 4000–400 cm⁻¹ and a resolution of 4 cm⁻¹. To illustrate the effect of Ag⁺ on the structural variations in EPS more clearly, each of the spectra was adjusted by subtracting the spectrum without Ag⁺ addition. 2DCOS analysis was performed using 2Dshige software (Kwansei-Gakuin University, Japan). Prior to 2DCOS analysis, the FTIR spectra were normalized and denoised by Savitzky-Golay smoothing. More detailed information on the mathematical procedures associated with 2D-COS can be found in the Supporting Information (SI-2).

3. Results and discussion

3.1. EAB resistance to Ag⁺ toxicity at various anode potentials

The mixed-culture EABs were grown by inoculating effluents from a well operated BES, where the current response was recorded by a potentiostat. As shown in Fig. 1a, the currents under various anode potentials cycled over time after refilling the reactor with fresh substrate. Stable currents were achieved from the third refilling, which indicated that the biofilms had become mature. The SEM of the EABs showed the homogeneous growth of microorganisms on the graphite plates under all anode potentials (Fig. S2).

The maximum current density of the EABs was found to depend on the applied anode potential. At 0.2 V, the EAB yielded a current density of 2.7 ± 0.3 mA/cm², which was higher than that at 0 V (2.5 ± 0.2 mA/cm²) and 0.2 V (2.2 ± 0.1 mA/cm²) and 0.4 V (1.9 ± 0.1 mA/cm²). Similar current responses were also observed from the CV scans under turnover conditions (Fig. 1b). A previous study also demonstrated that the EAB at 0.2 V yielded the highest catalytic activity among investigated EABs (Li et al., 2017). The underlying mechanism may be that an anode potential of 0.2 V could benefit EAB metabolic activity due to the difference between the anode potential and the standard biological redox potential of the substrate (Li et al., 2017). Besides, the amount of the EABs biomass under different anode potentials was in high accordance with the variation of current densities, which was consistent with the previous finding by Molenaar et al. (2018). The highest amount of biomass was obtained for the EABs formed at 0.2 V than those at other anode potentials (Fig. S1), suggesting that this potential is favorable for the formation of biofilm as well.

The mature EABs were further investigated for their resistance to Ag⁺ shock. However, after being exposed to Ag⁺ solution for 3 h, the current recovery ratios were 3.2%, 40.0%, 18.5% and 15.8% for the EABs corresponding to −0.2, 0, 0.2, and 0.4 V, respectively (Fig. 1c). Interestingly, a maximum current density of 1.0 ± 0.1 mA/cm² was obtained from the EAB formed at 0 V, which was significantly higher than those at other potentials. These results indicated that the EAB at 0 V, not that at 0.2 V, had the highest antishock capability. Notably, no significant decrease in current was observed when the same EAB at 0 V was treated in a KNO₃ solution (Fig. S3), suggesting that the decrease in current resulted from Ag⁺ exposure, not the operation process (i.e. moving electrode and oxygen exposure) or toxicity of NO₃⁻. In addition, the antishock properties of the EABs were further evaluated by exposing the EABs to Ag⁺ with different concentrations and duration periods. As shown in Fig. S4, the current recovery of the EAB formed at 0 V was the least affected by the low concentration of Ag⁺ and short duration period.

To further confirm the adverse effect of Ag⁺ shock, the viability of the EABs was analyzed with fluorescent staining to distinguish live versus dead cells. As shown in Fig. 2a, all the original electrodes were covered by the stained EABs with a varied thickness from 45 to 60 μm, which are similar to those previously reported (Sun et al., 2015). The thickest biofilm was obtained at 0.2 V, indicating that the growth of exoelectrogens was favored at this potential. In addition,
a typical two-layer structure of the EABs (a live outer-layer and a dead inner-core layer) was observed, which was in agreement with EABs developed by other researcher (Sun et al., 2015). For all the EABs, cells on the top layer were dead (red in color) after treatment with Ag$^+$ solution. The ratios of live to dead cells before and after Ag$^+$ exposure was shown in Fig. 2b, which had a similar tendency to the recovery ratios of current. A viability of 39.2% was observed for the EAB formed at 0 V, which was also higher than other EABs, indicating that more cells survived in this EAB after Ag$^+$ exposure. Therefore, the EABs developed at 0 V displayed the highest Ag$^+$ resistance in terms of electroactivity and cell viability, even though the highest amount of biomass was obtained for the original EABs formed at 0.2 V (Fig. S1).

3.2. Anode-potential modulated the EPS production in biofilms

As previous studies have reported, EPS play important roles in protecting biofilms against external stress (Lai et al., 2018; Li et al., 2019; Wang et al., 2013; Xue et al., 2012). In this regard, the structure and components of the EPS were analyzed, and the values varied among the EABs formed at different anode potentials (Fig. 3a). A total amount of 92.5, 136.7, 122.1 and 109.5 mg EPS/g VSS could be produced from the EABs formed at −0.2, 0, 0.2, and 0.4 V, respectively. These yields were similar to those previously reported for EABs (Angelalaiciney et al., 2017; Stockl et al., 2019). At the lowest and highest potentials, electron transfer had to be balanced, which may have resulted in the lower levels of EPS production obtained at these potentials than at the middle potentials (Hirose et al., 2018). EPS can be divided into TB-EPS and LB-EPS with respect to their association states with bacterial cells (Cao et al., 2011). The amounts of TB-EPS and LB-EPS were also affected by the anode potential. The highest amounts of both LB-EPS (61.9 mg-EPS/g-VSS) and TB-EPS (74.8 mg-EPS/g-VSS) were produced in the EAB formed at 0 V. The results indicated that the anode potential is capable of modulating EPS production in the mixed culture biofilms, which was in good agreement with the effect of anode potential on EPS production in pure-culture Geobacter biofilms (Yang et al., 2019a).

Using the extraction method, proteins and polysaccharides were measured to be the predominant components in both TB-EPS and LB-EPS (Fig. 3b and c), which were homogenously distributed in the biofilms, as revealed by CLSM (Fig. S5). The amounts of polysaccharides in LB-EPS increased as the potential increased from −0.2 to 0.4 V, while the polysaccharides in TB-EPS only increased from −0.2 to 0.2 V. However, proteins and humic acid showed the highest amounts in both TB-EPS and LB-EPS at 0 V. The 3D-EEM results also verified the higher fluorescence intensities of aromatic protein-like and humic acid-like substances (Ex/Em = 245–260/330–356 and Ex/Em = 318–330/380–390, respectively) for the LB-EPS samples at 0 V (Fig. S6). A previous study observed that electrochemical cultivation promotes protein production in the EPS of pure-culture Geobacter sulfurreducens biofilm, considering that they were involved in the extracellular electron transfer (EET) efficiency (Stockl et al., 2019; von Canstein et al., 2008). The same reason must be why proteins dominate among the EPS secreted from mixed-culture EABs.

To better understand the microbial metabolism related to EPS biosynthesis, transport and export, we applied PICRUSt to predict the functional genes associated with the production and transport of predominant components of EPS (i.e., exopolysaccharides, adhesion proteins, alginate synthesis compounds, pili proteins and poly-$\gamma$-glutamate) and genes related to “TCA cycle”, “ATP production” and “extracellular electron transfer” from metagenomic samples of EABs formed at −0.2–0.4 V (Fig. 3d). Accordingly, the abundance of genes involved in adhesion proteins, alginate
Fig. 2. (a) CLSM of EABs formed under different anode potentials before and after Ag\textsuperscript{+} exposure (red represents dead cells and green represents live cells); (b) Cell viability of the EABs before and after Ag\textsuperscript{+} exposure. The asterisks (*) indicate significant difference of the current densities for biofilm after Ag\textsuperscript{+} shocking compared to the original biofilm (P < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 3. (a) TB-EPS and LB-EPS contents of the EABs formed under various anode potentials; extracellular polymers per unit biomass of electroactive biofilm at different anode voltages for TB-EPS (b) and LB-EPS fractions (c), respectively; (d) Relative abundance of predicted functional genes involved in EPS production by PICRUSt analysis.
synthesis (i.e., alginate O-acetyltransferase, alginate biosynthesis transcriptional activator and alginate O-acetyl transferase), and folyolypoliglutamate synthase in EABs at 0 V was higher than those of other EABs. The exopolysaccharide synthesis-related genes, including exopolysaccharide synthesis and exopolysaccharide biosynthesis protein, were highest in the EABs formed at 0.2 V, which could explain the highest exopolysaccharide amounts in these EABs. In contrast, the EABs at 0 V showed the lowest abundance of genes encoding alginate lyases. The release of alginate lyases can degrade EPS, allowing bacteria to disperse from EABs (Sutherland, 1999). The downregulation of alginate lyase in EABs formed at 0 V might be one of the reasons for the high concentration of EPS in these samples. The functional genes associated with the TCA cycle (malate dehydrogenase, fumarate hydratase, succinate dehydrogenase, 2-oxoglutarate dehydrogenase, aconitate hydratase, citrate synthase and malate dehydrogenase) and ATP production (adenylate kinase) in EABs formed at 0.2 V were higher than those in EABs formed at −0.2, 0, and 0.4 V. In addition, a greater abundance of genes of cytochrome C, electron carrier protein and pili protein were observed in EABs formed at 0.2 V. These results indicated more metabolic activities in EABs formed at 0.2 V, which was consistent with the observed higher current generation.

3.3. The importance of proteins and humic acid in LB-EPS for protecting EABs from Ag⁺ toxicity

It is worth mentioning that all EABs developed at different anode potentials had similar microbial communities. As shown in Fig. S7, all biofilms were dominated by Proteobacteria similar to G. sulfurreducens. This phenomenon was consistent with a previous study by Zhu et al. (2014). Accordingly, differences in antishock properties toward Ag⁺ exposure should not be determined by the microbial community due to the lack of significant variation in various biofilms.

To determine the function of EPS components in protecting the EABs, the correlations between current recovery and concentration of the EPS components from the Ag⁺-treated experiments were investigated. As shown in Fig. 4, the current recovery of EABs after Ag⁺ exposure was weakly correlated with the polysaccharides and DNA of the outer-layer LB-EPS and inner-layer TB-EPS (R²<0.21; P > 0.05). The results suggested that the polysaccharides and DNA of LB-EPS could have limited the influence on the anti-shock properties of the EABs. However, significant correlations (R² = 0.94 and 0.95, respectively; P < 0.05) were found between the current recovery of the EABs and the proteins or humic acid of the LB-EPS. Positive correlations (R² = 0.97 and 0.98, respectively; P < 0.05) between the proteins or humic acid of outer-layer LB-EPS and cell viability were also obtained (Fig. S8). Proteins and humic acid can complex on the surface of solids to form porous, flexible and lipophilic LB-EPS (Whitfield et al., 2015; Yuan et al., 2017). It is also well known that proteins and humic acids are rich in chemical moieties that can bind with metal ions (Wei et al., 2017). Additionally, Wei et al. (2019) recently found that the proteins in sludge EPS, followed by humic acid, exhibited a relatively high adsorption capacity for metal ions whereas the lowest adsorption capacity was observed for polysaccharides.

Notably, stronger positive correlations were obtained between the proteins and humic acid of outer-layer LB-EPS and current recovery than between those of inner-layer TB-EPS and current recovery. The reason for this result could be that metal ions will first bind with outer-layer LB-EPS. Once the binding sites of LB-EPS are occupied, Ag⁺ will continuously bind with the proteins and HA of inner-layer TB-EPS. SEM-EDS analysis also demonstrated that a large amount of Ag⁺ accumulated in the EABs after they were exposed to Ag⁺ solution (Fig. S9). Therefore, the proteins and humic acid in LB-EPS could be the main components that react with Ag⁺ as a physical barrier to weaken its toxicity toward bacterial cells.

3.4. Fluorescence and FTIR hetero-2DCOS revealed the mechanism of EPS binding with silver

Considering that the above-revealed LB-EPS played a key role in protecting the EABs, the LB-EPS synthesized at 0 V under various Ag⁺ concentrations were selected to investigate the binding mechanisms. 2DCOS analysis which integrates fluorescence and FTIR spectroscopy has been proven to be a promising method to elucidate metal-organic interaction mechanisms (Chen et al., 2015b). By using this hetero-2DCOS analysis approach, fluorescence and FTIR signals can reflect the variations in different functional groups caused by Ag⁺, while synchronous and asynchronous 2DCOS analysis can help to better understand the relative direction and sequential orders of structural variations. Prior to 2DCOS analysis, general fluorescence and FTIR were used to achieve a reliable determination of the conformational changes in EPS induced by Ag⁺ binding. Fig. 5a shows the response of the fluorescence spectra of LB-EPS to the addition of different concentrations of Ag⁺. Three fluorescence fractions, including the protein-like region (250–300 nm), fulvic-like region (300–380 nm), and humic-like region (380–530 nm), could be roughly assigned on the spectra. Compared with the fulvic-like peaks and the humic-like peaks, the fluorescence intensity of protein-like peaks was relatively high, indicating a high proportion of proteins in the LB-EPS. As the concentration of Ag⁺ increased, the fluorescence intensities of the protein-like fractions and humic-like fractions weakened, which indicated the occurrence of electronic structural changes in the fractions due to binding with Ag⁺. The predominant infrared spectral characteristics of LB-EPS occurred in the range of 1000–2000 cm⁻¹, where we could detect almost all the vibrational information on the component backbone of EPS (Yu et al., 2011). Fig. 5b shows that the predominant peaks of LB-EPS corresponded to O–C–O groups of polysaccharides (1080 cm⁻¹), carbonyl C=O groups (1400 cm⁻¹) of humic acids or proteins, N–H bending vibrations of amines (1600 cm⁻¹), and C=O stretching in secondary amides (amide I) (1652 cm⁻¹). It can be observed that the peak intensities of the N–H bending of amines and C=O stretching of amides decreased as the concentration of Ag⁺ increased. In addition, the bands at 1400 cm⁻¹ and 1080 cm⁻¹, which were assigned to carbonyl C=O (1400 cm⁻¹) in humic acids or proteins and C–O–C (1080 cm⁻¹) stretching in polysaccharides (Whitfield et al., 2015), respectively, became weaker with increasing Ag⁺ concentration. The results suggested that these groups, such as carbonyl C=O in humic acids or proteins, N–H bending of amines, and C–O–C of polysaccharides, contributed strongly to binding Ag⁺.

Then, 2D-fluorescence-COS and 2D-FTIR-COS analyses were performed using synchronous fluorescence and FTIR spectra with the Ag⁺ concentration as the external perturbation. The synchronous 2D-COS map for the synchronous fluorescence spectra of EPS in the 250–550 nm region showed a predominant auto peak centered at 280 nm and a small peak at 480 nm identified from the cross-peaks (Fig. 5c), which indicated that the interaction of Ag⁺ with humic acids was less significant than that with protein. These cross-peaks were positive, indicating that the spectral changes proceeded in the same direction (i.e., decreased) as the Ag⁺ concentration increased. Moreover, the asynchronous 2D-COS map for the fluorescence spectra (Fig. 5d) provided additional information on the sequential binding relationships between EPS components and Ag⁺. Positive cross-peaks (11/12: 265/235 nm) are located in the upper left corner of the asynchronous map. According to Noda’s rule (Noda, 2012), the change followed the order 280 nm > 480 nm, suggesting that Ag⁺ bound to EPS fractions in the following...
sequence: protein-like fraction > humic-like fraction. Moreover, the binding mechanism between Ag⁺ and EPS was further investigated by 2D-FTIR-COS analysis. Six peaks (1650, 1600, 1527, 1400, 1265, and 1080 cm⁻¹) were more distinctly observed in the synchronous maps (Fig. 5e) than the FTIR spectra (Fig. 5b) for LB-EPS at various Ag⁺ concentrations. The signs of the cross-peaks from the synchronous map are presented in Table S1. The peak at 1080 cm⁻¹ was shown to change most significantly, followed by the peaks at 1600 cm⁻¹ and 1400 cm⁻¹, and all the cross-peaks were positive. This result verified that C=O, carbonyl C=O, and N–H groups were the potential moieties that responded to Ag⁺ binding in LB-EPS. The asynchronous maps for 2D-FTIR-COS showed three positive and twelve negative cross-peaks (Fig. 5f and Table S1). The signs of the cross-peaks (Table S1) in the asynchronous spectra
reflect the order in which the corresponding functional groups interact with Ag⁺. Accordingly, the sequence order of the functional groups involved in binding with Ag⁺ could be established: N—H of amines (1600 cm⁻¹) > N—H deformation and C—N stretching in −C=O−NH of proteins (1527 cm⁻¹) > carbonyl C=O groups (1400 cm⁻¹) > C=O stretching in secondary amides (amide I) (1650 cm⁻¹) > C—O—C stretching in phenolic groups (1265 cm⁻¹) > C—O—C of polysaccharides (1080 cm⁻¹).

Previous research confirmed that EPS from Escherichia coli can entrap Ag⁺ as AgNPs to hinder intracellular penetration of Ag⁺ (Kang et al., 2014). EPS have also been reported to effectively bind several other heavy metal cations (such as Cu²⁺, Pb²⁺, Cd²⁺ and Zn²⁺) through their large content of chelating groups (e.g., amino, carboxyl, and phenol) (Nocelli et al., 2016; von Canstein et al., 2008; Wei et al., 2019), Wei et al. (2019) suggested that heavy metal sorption onto carbonyl C=O and aromatic C=C stretching in humic acid and proteins from EPS can be attributed to significant electrostatic interactions. Although the functional group abundance in EPS differs among bacterial strains (Sheng and Liu, 2011), the binding of EPS to toxic metals will give the biofilm enough time to undergo the physiological or metabolic changes necessary for alleviating the toxic effect of the metal (Nocelli et al., 2016). Therefore, we can conclude that proteins were the predominant species in the EPS responsible for binding silver to attenuate its toxicity, in which N and O functional groups were the most important interaction sites.

4. Conclusion

EPS is considered the barrier for toxic species penetration into cells, but their function in protecting EABs has rarely been revealed. This study demonstrates, for the first time, that the anode potential appeared to influence the functional groups of EPS and humic acids from EABs, and chemical analysis demonstrated that the proteins and humic acids of LB-EPS were the main effective components. After that, spectroscopy combined with 2D-COS analysis revealed that the sequence of EPS backbone in terms of silver binding followed the order of amine of proteins > carbonyl C=O groups > phenolic groups > C—O—C of polysaccharides. The anode potential shows potential for modulation of EPS production in EABs, which also primarily determines the rate of electron flow from electroactive bacteria to the electrodes and controls the theoretical metabolic energy available for microbial growth. Therefore, a balance between anti-shock capacity and EET efficiency should be considered in the future. Finally, the findings of this study have important implications for in-depth understanding of the protective mechanism of electroactive biofilms and will finally lead to the optimization of BES performance under various wastewater matrices. Considering that BES has been proven to be a promising and sustainable wastewater treatment technology, our findings can be useful for enhancing its application in future wastewater treatment and energy management.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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