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Electron transfer at microbe-humic substances interfaces: Electrochemical, microscopic and bacterial community characterizations

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ABSTRACT

Soluble and colloidal humic substances (HSs) involved in microbial extracellular electron transfer (EET) have been extensively investigated. However, little is known regarding EET at HS adlayers that are bound or complexed to the surface of natural solids. Here, we investigated EET process on seven types of HS adlayers by forming electroactive biofilms (EABs) and monitoring the biocurrent generation. The HS immobilized surfaces were carried out by firstly modifying glassy carbon surfaces via electrochemical reduction of aryl diazonium salts to create amino-functionalized surfaces, and then various HSs were covalently bonded to these amino groups via linkage molecules. The results showed that the HS adlayers formed from Leonardite, Elliott Soil and Pahokee Peat humic acids (HAs) facilitated EET by forming more active EABs, whereas the Suwannee River and Aldrich HAs, Suwannee River and Pahokee Peat fulvic acids (FAs) hindered EET. The EET positively correlated with electron accepting capacity and wettability of the HSs, and negatively correlated with the polarity of the HSs, and only weakly correlated with electron donating capacity and zeta potential of the HSs. Microscopic images showed that the thickness and viability of the biofilms varied based on the HS properties. A microbial community structure analysis showed that all of the biofilms were dominated by *Proteobacteria* and selectively enriched in *Firmicutes* and *Bacteroidetes*. Our study indicated that the HS adlayers significantly affected microbial EET, and these results provide a more comprehensive understanding of the HSs involved biogeochemical and biotransformation processes.

1. Introduction

Humic substances (HSs) that are normally divided into humic acids (HAs), fulvic acids (FAs) and humin, are important components of natural organic matter (Klüpfel et al., 2014). In recent years, evidence has accumulated that HSs, which rely on their redox activity, can promote microbial extracellular electron transfer (EET) that plays a significant role in biodegradation of pollutants in wastewaters and transformation of minerals in soils (Van der Zee and Cervantes, 2009; Wang et al., 2009; Workman et al., 1997; Zhou et al., 2014). In these processes, HSs can accept electrons from a widespread variety of microorganisms that are capable of performing EET (known as exoelectrogens), such as iron reducing bacteria, archaea, denitrifying bacteria, and even methanogenic microorganisms (Martinez et al., 2013), and the reduced HSs donate electrons to minerals or other spatially distant electron acceptors that are not directly accessible to bacterial cells (Lovley et al., 1996; Lovley and Blunt-Harris, 1999). The redox chemistry of HSs and HS-mediated microbial EET has attracted attention over the past two decades, but studies have primarily focused on the electron shuttling capability of soluble HSs (Nurmi and Tratnyek, 2002; Scott et al., 1998). Their redox activity has been mainly attributed to the presence of a variety of redox-active functional units in HSs structure, such as quinone, phenolic hydroxyl, and N- and S-containing moieties (Aeschbacher et al., 2010; Ratasuk and Nanny, 2007).

In the environment, HSs are present as two types of solid forms. The first of these, which is predominate in the cores of soil particles, has been described as a rigid, inflexible and "hard" state humin (Weber et al., 1992). Similar to soluble HSs, solid humin has also shown remarkable redox activity for mediating microbial EET of microbial reductive dehalogenation (Zhang and Katayama, 2012; Roden et al., 2010). In contrast to humin, HSs can also bind or complex on the surface of solids to form porous, flexible and lipophilic "soft" adlayers (Weber et al., 1992; Bogan et al., 2003). The thickness of the "soft" HS phase can be up to 10 nm on soil particles and 20 nm on a carbonaceous surface (Bogan et al. 2003; Armanious et al., 2014; Armanious et al., 2016; Liu et al., 2011c). A variety of organic functional groups of HSs, such as carbonyl, carboxylate, phenol, and hydroxyl groups, must contribute to the high affinity of HSs for various water exposed solid surfaces to form HS adlayers on natural solid surfaces (Avena and Koopal, 1999). As a result, the HS adlayers interact with pollutants, enzymes, and cells in solution, which alter their fates and activities (Hong et al., 2015; Yuan et al., 2008;

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Sander et al., 2012). For instance, HS adlayers can alter the surface properties of clay minerals, which affect the bacterial interactions at the solid-liquid interfaces and the mobility of bacterial cells (Murphy et al., 1990). In addition, the HS adlayers immobilized on various solid surfaces, such as anion exchange resins, alumina particles and anaerobic granules, were capable of enhancing the biotransformation of pollutants in wastewaters, which represents advanced biological wastewater treatment techniques (Cervantes et al., 2011; Alvarez and Cervantes, 2012; Alvarez et al., 2012; Cervantes et al., 2015; Cruz-Zavala et al., 2016). In these cases, the HS adlayers have been suggested to play active roles in redox reactions catalyzing microbial conversion of pollutants. However, a comprehensive study of microbial EET process that occurs at the HS adlayers has remained largely unexplored, which is partly due to a lack of suitable methodologies.

The surface properties (i.e., charge and hydrophobicity) of the solids are influential on microbial EET because they affect the development of the biofilms (Guo et al., 2013; Santoro et al., 2015). The goal of this work was to assess how the HS adlayers affect the interactions of microbial cells with solid surfaces in terms of the attachment of exoelectrogens and the efficiency of microbial EET. To this end, seven different HAs and FAs spanning a wide range of physicochemical properties and originating from both terrestrial and aquatic sources were covalently immobilized on chemically well-defined electrode surfaces to mimic natural HS adlayers on solids. The electrode was selected as the model solid because it allowed for real-time monitoring of the EET using various electrochemical methods. The HS immobilized surfaces were assessed using scanning electron microscopy (SEM) and atomic force microscopy (AFM). The effects of the HS adlayers on the EET were systematically investigated using electrochemical, microscopic, and biological methods.

2. Materials and methods

2.1. Chemicals

Standard HAs and FAs from the Suwannee River (SRHA and SRFA), Pahokee Peat (PPHA and PPFA), Elliott Soil (ESHA), and Leonardite (LHA) were purchased from International Humic Substances Society (IHSS). Aldrich humic acid (AHA) was obtained from Sigma-Aldrich (St Louis, MO). The physicochemical properties of the HSs are listed in Table S1 of the Supporting Information. All materials were used as received.

2.2. Formation of the HS adlayers on the electrodes

The working electrodes were made of glassy carbon (GC) plates (10 mm × 10 mm × 2 mm) (Yueci Electronic Technology Co., Ltd., China) for HSs immobilization. Prior to use, the GC plates were treated as suggested by Guo et al. (2013). Briefly, titanium wires were glued to the back of the GC plates using silver paint to allow for a low-resistance external connection. Afterward, the back and sides of the plates were insulated with a coating of water proof epoxy glue, to give an exposed surface area of 1 cm^2. The modification of the HSs involved two steps (Fig. 1a). First, a sticky GC plate surface was fabricated via electrochemical reduction of in situ generated aryl diazonium cations in a 0.1 M aqueous HCl solution containing 10 mM 4-phenylenediamine (H_2N-Ph-NH_2) and 20 mM $NaNO_2$ (Liu et al., 2011a). The surface derivatization was then performed via scanning over a potential range between +0.6 and −1.0 V versus SCE for two cycles at a scan rate of 100 mV/s in an electrochemical cell. After the GC plate surface was derivatized with 4-aminophenyl (GC-Ph-NH_2), the plates were rinsed with Milli-Q water and dried under a stream of N_2 gas. Second, the resulting GC-Ph-NH_2 electrodes were dipped into a solution of 1 mg/mL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 2 mg/mL N-hydroxysuccinimide (NHS) for 1 h. Thereafter, 0.1 mg/mL of HSs was added into the EDC/NHS solution and allowed to react for 24 h at 4 °C. Stable HS adlayers formed on the surface through conjugation of the HS carboxyl groups to the primary amine group of the GC-Ph-NH_2 with the aid of EDC/NHS (Xu et al., 2004). The resulting HS modified GC plate surfaces were termed as GC-x (x represents the type of HS).

2.3. Characterization of the HS adlayers

The modified GC electrodes were analyzed using several surface techniques to check for successful formation of the HS adlayers. The surface hydrophobicity was obtained by measuring the contact angles with Milli-Q water using a Rame-Hart 100-00 goniometer. An average contact angle was achieved by measuring three different spots per substrate on three different substrates. Atomic force microscope (AFM) images were collected using a LEO1530VP AFM (Bruker Multimode 8, Germany). Scanning electron microscopy (SEM) images were collected using a Hitachi S-4700 microscope (Hitachi, Ltd., Tokyo, Japan).

2.4. Biofilm growth on the HS electrodes

To form electroactive biofilms (EABs) on the electrodes covered with various HS adlayers, a single chamber bioelectrochemical system (BES) was constructed as previously reported (Guo et al., 2013). A cylindrical container 14 cm in diameter and 8 cm tall (1000 mL) was used as the BES reactor. In the reactor, a saturated calomel electrode (SCE) reference electrode was placed in the center, eight working electrodes were arranged in a circle with the HS adlayers facing the reference electrode, and a titanium mesh surrounding the working electrodes was used as the counter electrode. The reactors were run in triplicate. The electrodes were connected to a multichannel potentiostat (CHI 1000C, Chenhua Instrument, China). The BES reactors were inoculated with 50 mL of preacclimated bacteria from another BES (originally inoculated with activated anaerobic sludge, Liede Sewage Treatment Plant, Guangzhou, China) and filled with a medium containing 1.0 g L^{-1} of sodium acetate in a 50 mM phosphate buffer (pH 7.0, PBS) supplemented with a 10 mL/L mineral solution and a 10 mL/L vitamin solution. A constant potential of 0 V vs. SCE was applied to each working electrode to monitor the biofilm formation. Cyclic voltammograms (CVs) of the biofilms were scanned in a potential window between −0.8 and +0.2 V (vs SCE) at a scan rate of 5 mV/s under both turnover (in the presence of acetate) and non-turnover (in the absence of acetate) conditions.

2.5. Structure and microbial community analysis of the biofilms

The morphologies of the biofilms formed on the GC plates were characterized using SEM (Yuan et al., 2013). The LIVE/DEAD BacLight viability kit (L7012, Thermo Fisher Scientific, USA) was applied to identify the bacteria viability using a laser confocal scanning microscope (CLSM, Leica TCS-SP2, Germany). The confocal images were captured along the biofilm thickness (Z axis) at regular intervals (0.5 μm) and were followed by a 3D volume reconstruction. The bacterial community of the biofilm that grew on the HS electrode was analyzed using Illumina sequencing. The DNA was extracted using a commercial kit (PowersoilTM DNA isolation kit; MO BIO Laboratories Inc., CA). The bacterial 16S rRNA genes were amplified with the universal primes 515F and 806R (variable region 4) and sequenced (Novogene). The pyrosequencing and bioinformatics analysis were performed according to a previously described method (Zhuang et al., 2015). Briefly, the paired-end reads were merged into single, longer sequences using FLASH. Then, the low-quality merged sequences (average quality value of 6) were removed from the downstream analysis. The pyrosequence reads were analyzed using a QIIME (Quantitative Insights into Microbial Ecology) v1.7.0 software package and UPARSE pipeline with the default parameters. The UPARSE pipeline was used to select the operational taxonomic units (OTUs) at the 97% sequence identity level. The