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In situ characterization and analysis of extracellular polymeric substances of *Pseudomonas fluorescens* biofilms in the presence of acidic electrolyzed water (#227)

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Introduction

Against the increase of bacterial resistance, acidic electrolyzed water (AEW) is considered as promising alternative. However, bacteria in adhered biofilms (AB), including the detached biofilms (DB) triggered by environmental changes, are more resistant to disinfectants than the planktonic cells. Extracellular polymeric substances (EPS) plays an important role in biofilm adhesion to surfaces and resistance to disinfectants. A better understanding of tolerance action can be possible by gaining more insight into the changes of composition and content of EPS inside the biofilms induced by AEW. Such a goal is possible by probing and tracking in situ the changes combining Raman and ATR-FTIR spectroscopy and verifying with the chemical analysis. The aim of this work was to assess the potential of this physicochemical approach to in situ monitor the changes of EPS in *Pseudomonas fluorescens* adhered and detached biofilms induced by disinfectants.

Methods

AEW treatment

For AB, a single stainless-steel coupon containing biofilms was rinsed three times with sterile 0.9% NaCl solution. The coupons were subsequently immersed in sterile 0.9% NaCl as a control or 20 and 60 mg/L AEW solutions for 10 min. The coupons were then immersed in neutralizing buffer solution (phosphate-buffered saline containing 0.8% sodium thiosulfate, pH 7.2) to stop the bactericidal effects of AEW. After rinsing, the biofilms were gently scraped from coupons into sterile 0.9% NaCl solution as DB, followed by their exposure to sterile 0.9% NaCl solution as a control or 20 and 60 mg/L AEW for 10 min. Neutralizing buffer solution was subsequently used to stop bactericidal effects.

Raman analysis

Raman spectra were acquired with a laser (633 nm) for excitation. Light was focused onto the sample through a 50× objective. The wavenumber range from 2000 to 400 cm^{-1} with the accumulation of 5, an acquisition time of 60 s and a grating of 600 lines/mm were collected for each sample.

ATR-FTIR analysis

ATR-FTIR spectra from 2000 to 800 cm^{-1} were acquired with an ATR-FTIR spectrometer with 2 cm^{-1} spectral resolution and 32 scans. A 300 µL aliquot of DB suspensions was transferred to the ATR crystal, and then transmission spectra were recorded as mentioned above. Appropriate spectra were used to remove the spectral background: a NaCl solution spectrum for DB and a stainless steel plate spectrum for AB.

Chemical analysis

Loose EPS (L-EPS) and bound EPS (B-EPS) were extracted using EDTA method. Cells were centrifuged and the supernatants were filtered through a 0.22-µm membrane, resulting in filtrate representing L-EPS. For B-EPS, the sediment was adjusted to a final cell density of ~5 mg wet-cell-biomass/mL. An equal volume of 2% Na_2-EDTA was added to the cell suspension, and the mixture was incubated at 4°C for 3 h. The cells were pelleted by centrifugation and supernatants were filtered through a 0.22-µm membrane. EPS were purifed using 3.5 kD dialysis membrane. The carbohydrate content was measured using phenolsulfuric acid method, and protein content was measured using Pierce BCA protein assay kit.

Results

Raman analysis

The Raman spectra of AB was significantly different from that of DB (Figure 1), which means a different chemical composition between two kinds of biofilms. AB have more richer bands which represent protein and carbohydrate. For one kind of biofilm, there were some difference in the variation of spectra after treatment of 20 and 60 mg/L AEW. Some characteristic bands were weakened or disappeared, especially after the treatment of 60 mg/L of AEW. For AB, we found that the peaks near the bands of 855-878 cm^{-1} were absent in all spectra of treatment groups.

ATR-FTIR analysis

For DB, spectra of all treatments displayed slight signals in the range from 1200 cm^{-1} to 2000 cm^{-1}, however the spectra of AB showed several more intense and significant peaks (Figure 2), indicating that there were many compounds in AB. The bands of 1548 cm^{-1} and 1647 cm^{-1} were assigned to proteins, and the bands of 1400, 1453 and 1237 cm^{-1} were associated with fatty acid, proteins, and phosphorus-containing carbohydrates. These substances were important component of mature biofilm. From the variations in biofilm spectra with the treatment of AEW, we found that the decrease of EPS was concentration-dependant, especially for bands associated with carbohydrate and proteins.

Chemical analysis

AEW treatment had a remarkable effect on EPS consumption, with significant difference ($P<0.05$) in protein and carbohydrate contents of the EPS in both biofilms, especially those of B-EPS (data not shown). We found that the protein content was higher than the carbohydrate content in the EPS of both biofilms. Cells in DB had insufficient EPS protection than AB, meaning less limited diffusion and a greater penetration of AEW. More antimicrobic ingredients can get inside the cells, thereby leading to more cell death.

Notes

Conclusion

According to our results of spectra and chemical analysis, DB and AB have different contents and composition of EPS The protein and carbohydrate were decreased by AEW, especially those of DB, which could lead to different extent death. These findings will be instructive for the important targets of biofilm control.

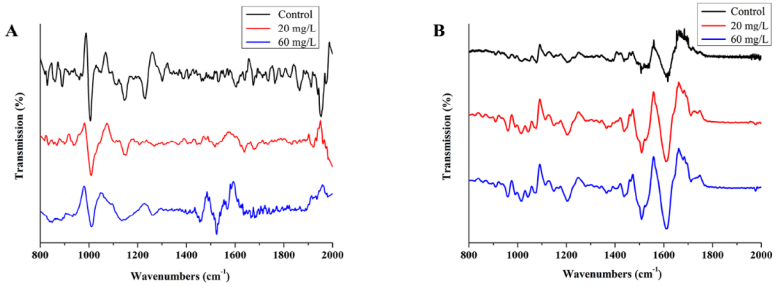
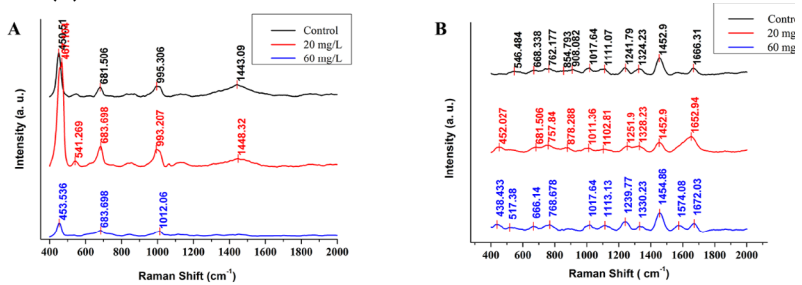


Fig. 1
Raman spectra of Pseudomonas fluorescens (A) detached biofilms and (B) adhered biofilms.

Fig. 2 ATR-FTIR spectra of Pseudomonas fluorescens (A) detached biofilm and (B) adhered biofilms



Notes