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# Redox modulation and non-invasive evaluation of phenotypic adaptation of *Escherichia coli* Biofilm

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Redox condition is a critical determinant of the metabolism of microbial biofilm. In this study, redox control of *Escherichia coli* biofilm adaptation in anaerobic chamber of electrochemical quartz crystal microbalance was performed to investigate the relationship between biomass yield and redox adapted metabolism. The electrodes on which biofilm grew were artificially controlled at 0 V, 0.2 V, 0.4 V (vs. Ag/AgCl, saturated KCl) and natural condition when no redox control was exerted. Meanwhile, mass of biofilm was non-invasively monitored. Biomass yield was larger in controlled redox conditions than natural condition. Real-time electric current was recorded, which suggests that metabolism via extracellular electron transport was enhanced in controlled redox conditions. The enhanced metabolism as a result of redox adaptation was consistent with the trend of biomass increment. Moreover, cellular redox potential was determined during biofilm development, suggesting that adaptation of cellular redox potential is necessary but not sufficient to give rise to metabolic adaptation.

**Keywords:** biofilm adaptation, redox control, electrochemical quartz crystal microbalance (EQCM), extracellular electron transport, cellular redox potential

## 1. INTRODUCTION

Microorganisms have strong propensity to attach to solid surfaces where they aggregate and proliferate in the form of biofilm [1]. However, the regulatory factors that dictate biofilm formation have not been fully understood. There are a variety of environmentally physiochemical cues that trigger bacterial adaptation to biofilm state and alter their metabolism [1]. One of the strongest regulatory factors is the environmental redox condition. Previous studies have shown that the metabolism and physiology of microbial biofilm is critically dependent on the redox condition [2-6].

Therefore, it is likely to control the biofilm formation by tuning up the redox conditions [7-10]. However, previous studies use chemical substances such as oxygen and hydrogen gas to control the redox condition. We realize that the use of chemical redox controller on one hand may complicate the adaptive response since chemical substances may act as chemoattractant and/or signaling molecules. On the other hand, the range of redox potential is limited by the electrochemical nature of the chemical substances; hence the extracellular redox condition could not be arbitrarily tuned. Moreover, the gaseous redox controller such as oxygen would not remain stable in aqueous medium. Therefore, the redox condition could not be maintained stable at a specific value.

In this study, instead of chemically controlling the redox condition, we used a computerized potentiostat by which the extracellular redox condition could be arbitrarily chosen and maintained by the electronic feedback loop. Although electronic control of the redox condition has been recently employed in bulk electrolysis system, the yield of biofilm on the electrode could not be non-invasively evaluated [11]. It is informative to obtain the history of biomass increment corresponding to redox adaptation to gain a holistic understanding of bacterial biofilm development [12]. The advancement of non-invasive tools such as quartz crystal microbalance (QCM) [13], surface plasmon resonance [14] and time-lapse image analysis [15] allow us to monitor the biomass or cell number of biofilm. Nevertheless, these tools alone could not control the redox condition and hence could not give information on how biofilm responds to different redox conditions.

To this end, we have for the first time [16, 17] introduced quartz crystal microbalance (EQCM) [18, 19] to combine the advantages of electronic redox control and non-invasive mass sensor to study the adaption of *Escherichia coli* (*E.coli*) biofilm in different redox conditions. In this study, systematic evaluation of the mass of biofilm that grew on the working electrode was performed through non-invasive monitoring of the mass increase in 0V, 0.2V and 0.4V (vs. Ag/AgCl, saturated KCl) redox conditions controlled by potentiostat. The electric current in the controlled redox conditions was monitored in real-time to investigate the extracellular electron transport activity. Meanwhile, cellular redox potential was recorded in-situ to understand how the cellular redox potential adapted in different redox conditions. The control experiment was performed without any redox control to mimic biofilm adaptation in natural anaerobic condition. The results of mass of biofilm in different redox conditions show the biofilm growth with metabolism via extracellular electron transport as a result of redox adaptation that would not occur in natural anaerobic condition.

## 2. EXPERIMENTAL PART

### *2.1. Microorganism preparation*

*E. coli* was aerobically grown in Luria-Bertani (LB) medium (Peptone, 10 g/L; yeast extract, 5 g/L; NaCl, 10 g/L) at 30 °C in a shaker incubator (XHZ-3, chem-star instruments) at 150 rpm. All chemicals are analytical grade from Sigma Aldrich. The growth of *E. coli* was monitored by Optical Density at 600 nm ($OD_{600}$ ) using Ultrospec 4300 pro UV/Visible Spectrophotometer (GE) with cuvettes of 1-cm path length. Culture was harvested at mid log-phase and diluted with fresh LB

medium. Bacterial concentrations were adjusted for each experiment to give a final concentration of $OD_{600}$ 0.3. The culture was immediately injected into EQCM chamber to start experiment.

### *2.2. EQCM as redox controller and monitor*

An EQCM analyzer (CHI 440A, CH Instruments) was used to control the potential on the working electrode on which biofilm was growing. The electrode potential was maintained at 0 V, 0.2 V and 0.4 V (vs. Ag/AgCl, saturated KCl) to modulate the extracellular aquatic redox condition by the potentiostat module of EQCM. During the course of biofilm adaptation, cellular redox potential was monitored in-situ by temporarily suspending the redox control and recording the open circuit potential on the working electrode against the reference electrode. The control experiment was operated without redox control to mimic natural biofilm development.

### *2.3. Electrochemical cell configuration*

To control the redox condition and monitor the redox potential of bacteria, a three-electrode configuration was adopted. The gold-coated quartz crystal electrode (diameter 6 mm, CHI 125A, CH Instruments) was served as the working electrode. Pt wire (CHI 129, CH Instruments) and Ag/AgCl (saturated KCl, CHI 128, CH Instruments) were used as counter and reference electrode, respectively. Prior to use, working and counter electrodes were cleansed with piranha solution (70% $H_2SO_4$ and 30% $H_2O_2$) and thoroughly rinsed with Milli-Q water. Reference electrode was sterilized by 75% ethanol solution. Airtight and autoclaved chambers of identical configuration were used. All EQCM experiments were performed at 22 °C. All potentials in this study were referred to Ag/AgCl (saturated KCl). To evaluate *E. coli* biofilm development under anaerobic condition, before the EQCM experiment, nitrogen gas stream was used to purge the dissolved oxygen in bacterial culture.

### *2.4. EQCM as mass sensor*

Besides the role as redox controller and monitor, EQCM was also used to non-invasively evaluate the mass attached to on the surface of working electrode. The fundamental frequency of the pristine working electrode is 7.995 MHz. Foreign mass attached onto the electrode surface results in shift of resonant frequency from 7.995 MHz. Mass change ($\Delta m$) of surface layer is proportional to shift of resonant frequency ($\Delta f$) as depicted by Sauerbrey equation [20], and in a simplified form the equation can be written as

$$\Delta m = -A\Delta f \qquad (1)$$

Where $A$ is proportionality factor (1.34 ng $Hz^{-1}$) as determined by the intrinsic properties of electrode. Therefore, a negative shift of the resonant frequency denotes an increase of mass of the surface layer.

*2.5. Cell number determination*

To confirm the results of mass sensor, after the 150 h biofilm development experiment, the working electrodes were taken out of the electrochemical chambers and rinses three times with Milli-Q water to ensure that the remaining cells were firmly attached to the electrodes. The surface colonizing biofilm cells were then observed under Olympus BX51 microscope. Images were captured with 500 × total magnification. Planktonic cell density was also determined immediately after the 150 h experiment by taking liquid samples from the chambers. The cell density of the sample was determined by direct counting using Neubauer hemocytometer under Olympus BX51 microscope.

## 3. RESULTS AND DISCUSSION

*3.1. Non-invasive evaluation of the biomass attached to the electrode surface during biofilm adaptation in different redox conditions*

EQCM method takes the advantage of both QCM and electronic control of redox condition. The QCM module functions effectively as mass sensor based on the principle of reverse piezoelectric effect [20]. The working electrode that accommodates bacteria attachment is fabricated by AT-cut quartz crystal of which gold layers are coated on each side of the crystal disc. The gold-coated quartz crystal electrode is driven to oscillate in thickness shear mode by applied AC voltage. Only one side of the crystal electrode was exposed to solution, the other side remained pristine throughout the experiment (Fig. 1). Difference of the oscillation resonant frequency of the two sides could be recorded in-situ to reflect the mass attached to the side exposing to solution. The potentiostat module of EQCM enables us to control the potential on the working electrode at arbitrary physically meaningful value.

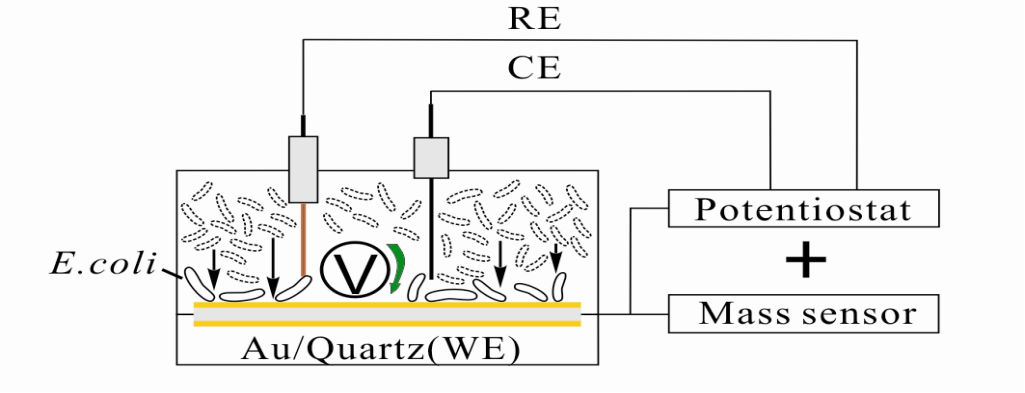


**Figure 1.** Schematic presentation of the mass sensor (QCM) and redox controller/monitor (potentiostat) modules of EQCM. The gold-coated quartz crystal electrode serves a dual function as mass sensor and working electrode (WE) for redox control and monitoring in conjunction with reference (RE) and counter (CE) electrode.

The EQCM experiments were operated in 0 V, 0.2 V, 0.4 V and natural conditions (details in methods). Experiments of each condition were repeated in triplicate. Good agreement among the results was obtained and the representative results were shown in fig. 2. When the *E. coli* culture was injected into the EQCM chambers at 0 h, the frequency began to decrease in all conditions (Fig. 2A). Since the negative frequency shift denotes an increase of mass on the electrode surface (Equation 1), these results suggest that more and more cells were colonizing on the electrode surface as time developed. It is notable that the final frequency shift is dependent on the redox condition. It is −473 Hz for 0 V, −675 Hz for 0.2 V, −356 Hz for 0.4 V and −184 Hz for non-controlled (natural) condition.

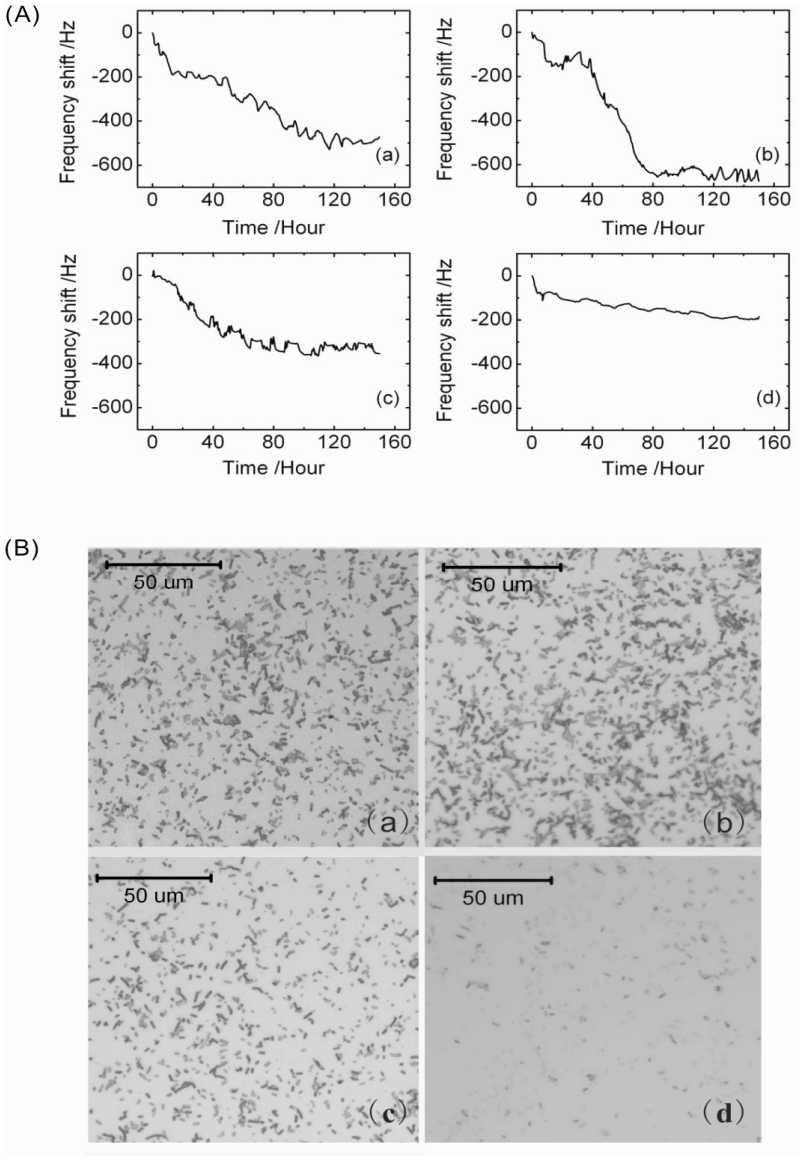


**Figure 2.** (A) Resonant frequency shift of the working electrode in 0 V (a), 0.2 V (b), 0.4 V (c) and natural (d) conditions. (B) Optical microscope images (500 x total magnification) of final (at 150 h) *E. coli* biofilm on electrodes in 0 V (a), 0.2 V (b), 0.4 V (c) and natural (d) conditions.

Interestingly, the final mass of biofilm is not linearly dependent on the redox potential. The biofilm grown at 0.2 V but not at 0.4 V exhibited the largest biomass (Fig. 2A.b). This result suggests the dual effect of the redox potential on biofilm development.

At the physiological condition *E. coli* cells carry negative charges, the more positive the potential on the electrode is, the higher probability it could attract more bacteria. Therefore, the electrode maintained at 0.2 V accommodated more cells than 0 V. However, as the potential became more positive at 0.4 V, adverse effect may arise. It was previously reported that cell growth and division could be retarded due to the strong binding force between the negatively charged cells and the positively charged surface [21]. That is perhaps why the biomass at 0.4 V is lower than 0.2 V and 0 V. So there is a tradeoff between the attraction force and inhibitory effect on cell growth in selecting the redox condition.

To verify whether the frequency shift indeed reflected the amount of bacteria cells attached to the electrode surface, ex-situ microscopic examination was performed. The electrodes were taken out of the EQCM chamber and thoroughly rinsed to ensure that the observed cells were firmly adhering to the surface. As shown in fig. 2B, the cell density is the highest for the EQCM experiment at 0.2 V. The microscopic result of cell density is in good agreement with the resonant frequency shift, confirming that the EQCM is an effective tool to evaluate the biomass development of the surface residing biofilm cells.

### *3.2. The density of planktonic cells in different redox conditions and the implication on metabolism*

Besides the surface residing biofilm, there were also considerable amount of planktonic cells growing in the bulk liquid. It is necessary to examine whether the density of planktonic cells was also relevant to the redox condition. Fig. 3A shows that the planktonic cell density did not differ much in controlled redox conditions (0 V, 0.2 V and 0.4 V).

Nevertheless, it is significantly higher than that in the natural anaerobic condition. In sharp contrast, the biofilm cell density as represented by biomass (calculated by Equation 1) was much more sensitive to the controlled redox conditions. The growth rate of bacteria is critically related to the cellular energy turnover. It is well known that all living organisms from microorganisms to human being are powered by adenosine triphosphate (ATP) that is generated by electrochemical gradient across cell membrane [22].

Therefore electrochemical gradient is critical to energetic budget of every living organism, which is maintained by donating electrons to terminal electron acceptor [23]. The most commonly used terminal electron acceptor is molecular oxygen in the mode of aerobic respiration. When oxygen is not sufficiently available some microorganisms could respire with alternative terminal electron acceptors such as iron [24] and sulfur [25]. Such anaerobic respiration coupled with the establishment of electrochemical gradient across cell membrane generates more ATP than fermentative glycolysis [26, 27]. In previous cyclic voltammetric study we have evaluated the mechanism of extracellular electron transport from *E. coli* to working electrode [28].

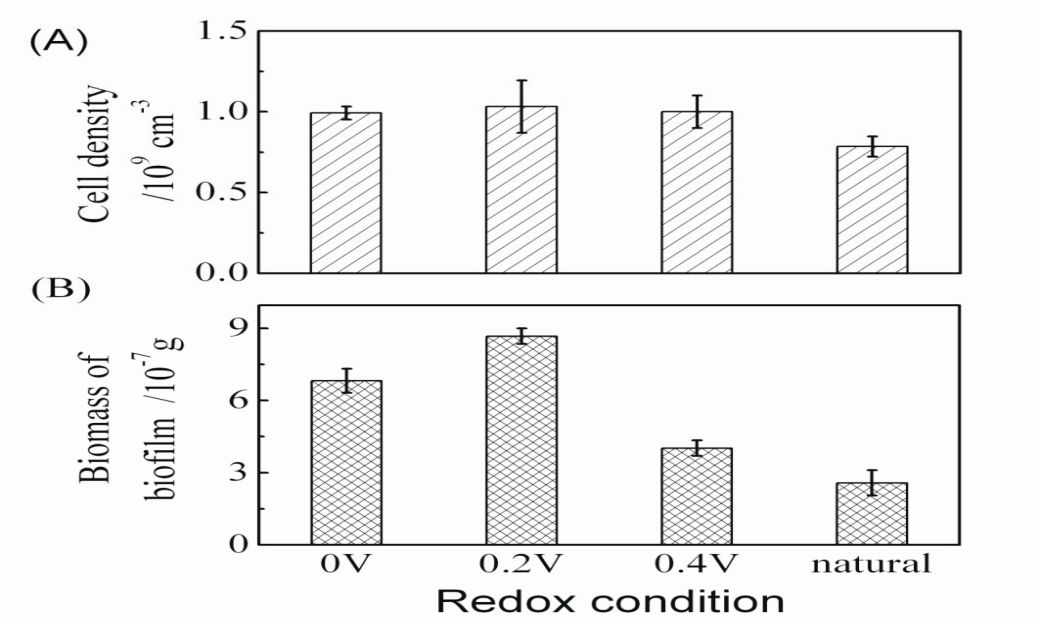


**Figure 3.** (A) Final (at 150 h) planktonic cell density measured by direct counting. (B) Final (at 150 h) mass of biofilm on electrodes in 0 V, 0.2 V and 0.4 V and natural conditions. Error bar represents standard deviation, n=3.

In the anaerobic condition of this study, *E. coli* could not use molecular oxygen as terminal electron acceptor. Instead, it could use electrode as alternative terminal electron acceptor and obtain higher energy turnover than fermentative glycolysis as in the natural condition. As a result, when potential was maintained at 0 V, 0.2 V and 0.4 V, *E. coli* grew much faster than that in the natural condition. The above results imply that *E. coli* cells have differentiated response to the redox conditions. Both the biofilm and planktonic cell density in the controlled redox conditions were significantly higher than that in the natural condition due to different mode of metabolism. Moreover, biofilm cells were more sensitive to the redox conditions than planktonic cells. Apparently, biofilm cells residing on the electrode were experiencing stronger electric field than planktonic cells.

### *3.3. Monitoring of the electric current in controlled redox conditions*

In addition to the history of biomass increment, complementary evidence on the adapted metabolic activity of *E. coli* under controlled redox conditions was shown in fig. 4A.a-c. As illustrated in fig. 4A.d, the observed electric current is the collection of electrons donated by *E. coli* via extracellular electron transport. Intriguingly, the electric current obtained at 0 V, 0.2 V and 0.4 V all demonstrated biphasic pattern. The electric current increased sharply at the early stages and then saturated at plateau. The biphasic behavior was also observed in the history of biomass development (Fig. 2A). These results suggest that at early stages bacteria enhanced metabolism via extracellular electron transport to the working electrode to provide energy for the growth of biofilm. At later stages, the metabolic rate as indicated by the electric current could not be further elevated; consequently, the

biomass ceased to increase. In future study, it would be valuable to unveil the regulatory circuits that controlled the biphasic behavior to gain better control over biofilm development.

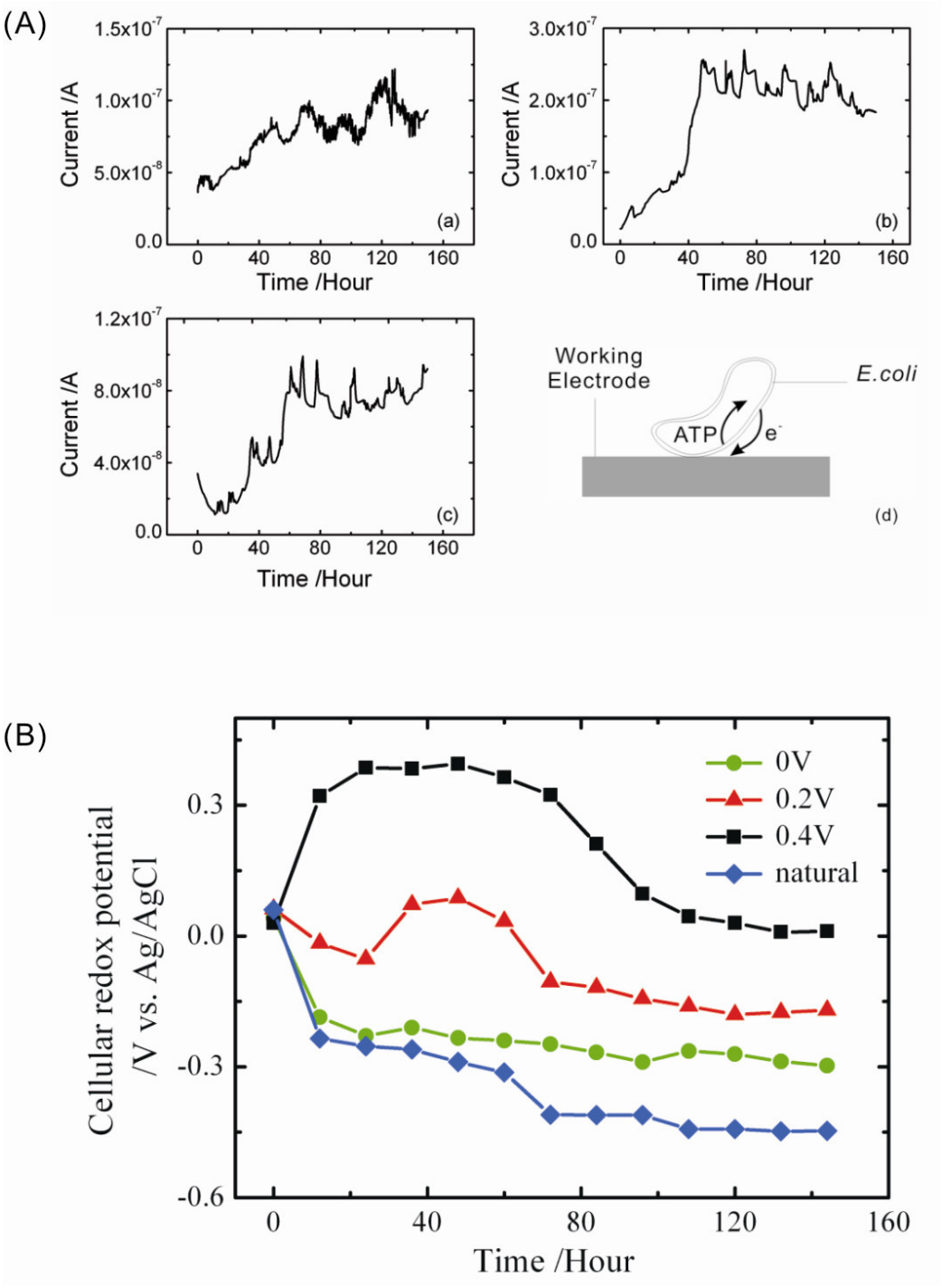


**Figure 4.** (A) Electric current in the controlled redox conditions: (a) 0 V; (b) 0.2 V; (c) 0.4 V. (d) Depiction of metabolism via extracellular electron transport. (B) Adaptation of redox potential of *E. coli* in 0 V (circle), 0.2V (triangle), 0.4 V (square) and natural (diamond) conditions.

*3.4. Adaptation of cellular redox potential in different redox conditions*

Noticing the intricate dependence of biofilm growth on the surface potential, we then carefully examined whether and how the redox potential of *E. coli* cells evolved with time. Starting with the same initial cellular redox potential (0 h) at 0.0485 ± 0.015 V (mean ± SD, $n$=4), the redox potential of *E. coli* has been monitored in-situ during the course of 150 h biofilm development in different conditions (Fig. 4B). When there was no control of the extracellular redox condition, biofilm naturally adapted under anaerobic condition. The redox potential of natural biofilm monotonously shifted negatively to a final potential of −0.455 V (Fig. 4B). In this case the redox potential of cells was coupled with that of their aquatic environment, thus the redox potential of *E. coli* is indistinguishable from its redox environment. Nevertheless, under controlled redox condition, where the redox condition has been maintained at 0 V, 0.2 V and 0.4 V throughout the course of biofilm development, the cellular redox potential was able to decouple from the environment.

*E. coli* grew at 0 V demonstrated the same monotonous trend as natural development and stabilized at −0.252 V finally. Slightly different behavior was observed at 0.2 V. Although biofilm adapted towards lower redox potential from 0 to 24 h, it reversed the trend from 24 to 48 h after which it resumed the initial trend and the cellular redox potential finally decreased to −0.234 V. Similar behavior was observed at 0.4 V. In this case the cellular redox potential increased to 0.395 V in 48 hrs but subsequently decreased to a final potential of 0.037V. Redox potential of *E. coli* biofilm exhibited pronounced decoupling from its environmental redox potential at 0 V, 0.2 V and 0.4 V. This reflects the thermodynamic nature of living organisms as far-from-equilibrium systems [29]. It is vital for microorganisms such as *E. coli* to maintain their redox homeostasis rather than to passively couple with redox environment [5, 30]. Although intensive attention has been paid on the subject of cellular redox regulation the dynamic network that dictates cellular redox potential is not fully understood. The redox regulatory circuit seems quite robust. The cellular redox potential insisted to adapt towards lower redox potential even at very positive (0.4 V) redox condition.

The reason of the common trend of cellular redox adaptation again embeds in cellular energetics. Under the anaerobic experimental condition, when environmental redox potential was not artificially controlled, cells were not able to donate electrons to external electron acceptors and they had to adopt fermentative glycolysis that generates less ATP and hence resulted in the smallest population size. When environmental redox condition was artificially controlled, cells were able to decouple from environment and exploit the redox gap between cells and electrode to generate energy via extracellular electron transport. It is known that this pathway generates more ATP than fermentative glycolysis under anaerobic condition. Therefore, higher energy gain under controlled redox conditions gave rise to larger biomass yield.

## 4. CONCLUSIONS

EQCM was demonstrated as a means to control and non-invasively monitor biomass and cellular redox potential of biofilm development. It enabled us to reveal the correlation between redox-

adapted metabolism and cell growth which could not be attained by other methods. The influence of environmental redox condition on biofilm development is intricate, and the largest biomass yield was observed at 0.2 V in this study due to the ambivalent effect of the positive potential on biofilm development. Larger biomass was obtained for both biofilm and planktonic cells in controlled redox conditions than non-controlled condition. *E. coli* obtained higher metabolic gain through extracellular respiration which could not be achieved under natural anaerobic condition. As indicated by the history of electric current during biofilm development in controlled redox conditions, environmental redox condition is likely to trigger a cascade of regulations of cell metabolism of which the detailed pathway deserves further study. Common trend of adaptation of cellular redox potential was found in all conditions where *E. coli* adapted towards lower redox potential. It is worth studying whether the robustness of redox regulation has an evolutionary origin.

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