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Development of Electrochemical Platforms Devised for Bacterial Activity Monitoring

Dung Quynh Le
June 2015

Doctoral Thesis at Osaka Prefecture University
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CHAPTER I

Introduction

1.1. Introduction of Bacteria

1.1.1. Roles of microorganisms

Microorganisms are widely found in nature; each bacterium may weigh solely $10^{-11}$ g but collectively they constitute about 60% of biomass in the earth. It is well known that microorganisms play an essential role in the life network. They are a component of many food chains, in which they create food or become food themselves for other organisms; they make nitrogen available for plants and plants, along with certain photosynthetic bacteria, make oxygen available for humans and many other organisms. They decompose dead organisms, or wastes from living ones, in that way, microorganisms maintain the balance in nature. Besides those natural activities, people, for years, utilized microorganisms in the food industries to produce beer, cheese, etc. and medical applications such as antibiotics, vaccines. More recently, a number of applications in environment-related fields, such as bio-remediation, industrial waste treatment proceeded under the participation of bacteria, which naturally love to eat contaminants or have been genetically modified to have the taste to toxins. In an attempt to produce fuels and energy through bacterial activity, many groups have developed bioelectrochemical systems, in which microbes are used to catalyze the reactions at the anode and/or cathode. Hydrogen and ethanol were generated in a microbial electrolysis cell, as the potential is applied to drive a nonspontaneous reaction; whereas electric current is generated, as bacteria transfer electron from soluble electron acceptor, such as oxygen, nitrate to anode, via membrane-bound components or soluble electron mediators in microbial fuel cells.

Yet less than 1% of identified microorganisms cause disease, most of the remaining directly or indirectly brings benefit to humans and the environment; pathogens remain a major public health challenge for years. Food-borne diseases (FBD) are not limited solely to developing countries; it is estimated that in Netherlands 80 people die each year because of FBD, while this rate is 2,612 in the United States. Pathogens can contaminate minimally processed foods, dairy products or fruits and vegetables; food animals and poultry or sea-food are their potential “homeland”. Among food-borne pathogenic bacteria, \textit{Escherichia coli} (O157:H7) and \textit{Salmonella} sp. are two of the most dangerous ones. A very low concentration
of 10-100 cells of these bacteria in food can cause severe damages, which may lead to death.\textsuperscript{16} In order to invade host cells, pathogens firstly need to attach to the cells, and then produce substances to facilitate their invasion. Once overcoming the defense and successfully invading the host cells, they cause diseases by (1) releasing toxic lipopolysaccharide components of their outer membrane (endotoxin), (2) releasing toxic proteins and enzymes (exotoxin), or (3) the competition with host cells in taking iron (siderophores). To protect themselves from the host defense systems, bacteria have evolved many tactics;\textsuperscript{17} some Gram-negative bacteria are secured to antibiotics thanks to the lipopolysaccharide layer on their outer membrane behaving like a tight barrier. Thus, bacterial infections and drug resistance remain the serious medical problems.

1.1.2. Bacterial cell structure and electron transport chain

There are many groups of microorganisms, including virus, bacteria, fungi, protozoa, and some algae. In the framework of this thesis, bacteria are the subject of study; therefore, all the information below is given for bacteria.

\textit{a/ Overview of cell structures}

Bacteria typically appear in three basic shapes: spherical, rod-like, and spiral, ranging from 0.5 to 2.0 µm in diameter.\textsuperscript{1} Bacterial cells almost always are bounded by a chemically complex cell envelope, which surrounds a matrix termed cytoplasm.

The cell envelope consists of outer and inner membranes; lying between these two membranes are the periplasmic space and peptidoglycan layer. The inner membrane is called plasma membrane or cytoplasmic membrane as surrounding of the cytoplasm. The peptidoglycan layer, periplasmic space, filling of periplasm, the outer membrane, other external components like pili, and capsules, if present, structure the cell wall of a bacterial cell (see Fig. 1-1).

The outer and inner membranes contain both lipids and protein in varying proportions. The membrane-associated lipids are structurally asymmetric with polar and non-polar ends; this property enables lipid to form a bilayer membrane. The outer surfaces are hydrophilic, while hydrophobic ends are buried in the interior. Proteins are either embedded in or loosely bound to the lipid bilayer; the former ones are insoluble in an aqueous solution and not easily extracted, while the latter ones are soluble and readily removed.
The cytoplasmic matrix contains 80% of water; the remaining 20% are substances dissolved or suspended in the water, including enzymes, proteins, carbohydrates, lipids and a variety of inorganic ions. Many chemical reactions, both anabolic and catabolic, occur in this matrix. The genetic material is localized in the discrete nucleoid, included in the cytoplasm with no membrane.

Based on their response to staining (Gram stain, 1884), bacteria were divided into two large groups; Gram-negative and Gram-positive. The reason is the difference in the structure of their cell wall; the Gram-positive bacteria have a thick peptidoglycan layer, which functions as the outer membrane or cell wall, while the Gram-negative species have a more complex cell wall, consisting of a thin peptidoglycan layer, periplasmic space, and an outer membrane (Fig. 1-1).

Figure 1-1. The cell envelope of a typical Gram-negative bacterium (adapted from Shiigi et. al, 2015)

b/ The lipopolysaccharide layer (LPS)

Besides lipids and proteins, the outer membranes of some bacteria, typically Gram-negative bacteria, contain a lipopolysaccharide layer (LPS), which plays an important role in their adhesion, transport and antibiotic resistance. LPS is also termed endotoxin due to its virulence once a bacterium has adhered to a host cell.

The structures of LPS are described in detail elsewhere and only a brief overview is provided here (see Fig. 1-1). LPS contains both lipids and carbohydrates, and comprises of three fractions: lipid A, the core oligosaccharide and the O-antigen. Since LPS is partially...
phosphorylated, the P group confers the net negative charge. The LPS is anchored to the outer membrane by the lipid A - a glucosamine-based phospholipid, which is known to involve in pathogenesis of bacteria cells. The core polysaccharide is divided into two parts, the inner and the outer cores. The inner core attaches directly to the lipid A and commonly contains sugars such as heptose and 3-deoxy-D-manno-octulosonic acid (or KDO). The outer core region provides an attachment site for O polysaccharide (O antigen). A sequence of repetitive subunits called O chain or O antigen is attached to the core oligosaccharide, and comprises the outermost domain of the LPS molecule. The composition of the O chain varies from strain to strain.

In an infected host cell, LPS is not released from the bacterial cells until their membranes are broken, e.g. due to antibiotic treatment. Small amounts of LPS can be protective by stimulating the host immune system; large amounts, however, induce high fever, decrease blood pressure, and arise in other toxic events that may cause to death.25

c/ Electron transport chain and proton pump

The electron transport chain, coupled with the proton pump, drives the energy production in all living organisms (Fig. 1-2), including bacteria. In addition, quinones, which are the crucial components of these processes, are a subject in the current study, so that the general mechanisms of electron transport and proton pump are briefly introduced below.

Bacteria “eat” food, and through metabolism pathways energy is taken from food and stored in high-energy molecules such as nicotinamide adenine dinucleotide (NADH) or flavin adenine nucleotide (FADH₂) in their reduced forms. These molecules then join and initiate the electron transport chain, often called the respiratory chain by providing their electrons and protons. The respiratory chain, embedded in the plasma membrane contains three protein complexes, ubiquinone and cytochrome c. In these complexes, as electrons are transferred along a series of protein-bound electron carriers, from a high energy level to a lower one, energy is released to pump proton outside the plasma membrane. Electrons are transferred between protein complexes by the mobile electron carriers ubiquinone and cytochrome c to complete the electron transport chain. Ubiquinone in the respiratory chain picks one proton for every electron it accepts and the proton is released as the reduced ubiquinone donates its electron; therefore, ubiquinone functions as a carrier of both electrons and protons in the respiratory chain. The pumping of protons from inside (cytoplasm) to outside (periplasm) the plasma membrane creates an electrochemical proton gradient between these matrices. This gradient is harnessed to make ATP (adenosine 5’- triphosphate), the energy “currency” of bacteria by the enzyme
complex ATP synthase, through which \( H^+ \) flows back into the cytoplasm; this process is termed oxidative phosphorylation or chemiosmosis and underlies function of all living organisms.\(^1\),\(^{18}\),\(^{27}\)

**Figure 1-2.** The general mechanism of electron transport and oxidative phosphorylation

1.2. Isoprenoid quinones

1.2.1. Occurrence and functions

In the previous section, we have seen the crucial role of the quinones in the electron transport chain as a carrier of electrons and protons between protein complexes, a brief introduction of their occurrence and functions is given herein.

Isoprenoid quinones, which are found in nearly all living organisms, are composed of a polar head group and a hydrophobic side chain. The hydrophobic part allows the quinones freely move in lipid bilayer, while the hydrophilic head groups enable the interaction with hydrophilic parts of proteins. Therefore, the quinones not only serve as a mobile electron carrier in the lipid bilayer between protein complexes but also permanently associate with proteins and transfer electron within these complexes.\(^{28}\)

The reduced forms of the isoprenoid quinones have antioxidant properties, which protect membranes from lipid peroxidation and deleterious effects of reactive oxygen species on the membrane components. Recently, a number of reports suggested the function of isoprenoid
quinones as the enzyme cofactor; these compounds were also proposed to participate in the regulation of gene expressions and signal transductions within cells.29

The great majority of the biological isoprenoid quinones belong to naphthoquinone and benzoquinone.30 In respect to the results of current work (chapter 4), which have suggested the participation of ubiquinones and menaquinones, these two representatives of benzoquinone and naphthoquinone groups will be reviewed.

Menaquinones (MKn) (n presents the number of the prenyl unit), which are the most widespread microbial respiratory quinones belong to the group of isoprenoid naphthoquinones (Scheme 1-1). MK has a low $E^{\infty}$ value, and their appearance, as the most ancient type of isoprenoid quinones, can be connected with the reducing character of the atmosphere, owing to the absence of molecular oxygen before oxygenic photosynthetic bacteria appeared. The isoprenoid side chain of MK is most frequently composed of 6-10 prenyl units and fully unsaturated; however, the length and saturation degree of the side chain often depend on the growth temperature of a given species.30

Scheme 1-1. Menaquinone (MKn)

Ubiquinone (UQn, Scheme 1-2) is a member of isoprenoid benzoquinone group and evolutionarily “younger” than MKn. The number of isoprenoid units frequently varies among species, and some have more than one type of UQn. In bacteria UQ8-10 are the most observed quinones, while UQ1-7 could also be found in minor amounts.30

Scheme 1-2. Ubiquinone (UQn)
1.2.2. Quinone profiling as a tool for microbial characterization

Isoprenoid quinones are lipid-soluble substances found in almost all species of organisms and locate in the inner membrane of bacteria. In addition to their crucial role as electron carrier coupling to proton translocation, quinones have attracted attention with their significance in microbial systems. Though the type of quinones varies with culturing conditions, each species is defined by certain quinones and this is relatively unchanged. The report of Hedrick and White in 1986 first time demonstrated the value of quinone analysis, in which the ratio of menaquinone/ubiquinone was possible to characterize the redox state of microorganisms. This biomarker approach, called the quinone profile method has been successfully developed for the determination of microbial communities in different environments, such as lake and marine surface sediments, waste materials, or soil.

Quinones are usually extracted from microbial cells by an appropriate organic solvent mixture, such as chloroform-methanol (2:1, v/v) or acetone. The extract is then evaporated under vacuum and treated for several steps with n-hexane or other organic solvents, following by chromatography or mass spectroscopy or both for separation and quantification.

Hiraishi and Kato took sediments sample of five lakes in Japan and conducted quinone profiling for determination of microorganism species dominated. The total quinone contents of these samples ranged from 1.97 to 18.0 nmol/g dry weight of the sediments, of which a combined fraction of ubiquinones and menaquinones accounted for 42 to 74%. The remaining fractions (26 to 58%) consisted of the photosynthetic quinones, plastoquinones and phylloquinones. The results of quinone type analysis using spectro-chromatography and mass spectrometry indicated the presence of inhabitant species, which carried ubiquinone-mediated aerobic respiration, oxygenic photosynthesis, and menaquinone-associated respiration.

In another study, the redox states and composition of quinone pool (lipid bilayer of inner membrane) of Escherichia coli was obtained during aerobic-batch culture growth by quinone profiling technique, using HPLC, UV/Vis spectral and mass spectral analysis. The presence of both oxidized and reduced forms of ubiquinone (UQ8), demethylmenaquinone (DMK8) and menaquinone (MK8) in quinone pool of E.coli cultured under anoxic batch condition was observed. The redox states and absolute components of the UQ pool changed negligibly in initial phase of insufficient oxygen cultures or highly aerated cultures; nevertheless, the UQ pool became more reduced upon entry into exponential growth phase, in which oxygen was largely consumed due to the rapid growth and respiration of bacteria. The total content of both
reduced and oxidized quinones also decreased, and along with this decrease was the synthesis of DMK8 and MK8. The results indicated that both redox state and composition of quinones pool vary according to oxygen availability.

1.3. Conducting polymers

1.3.1. Electrochemical polymerization of conducting polymers

Conjugated polymers have received extensive concern since the discovery of the electrical conductivity in polyacetylene by Shirakawa, Heeger and Mac Diarmid in 1977. The conjugated polymers enable charge conduction thanks to delocalized electrons along their backbones. Neutral polymers are either insulators or semiconductors. To make these polymers electrically conductive, electron acceptors or donors should be introduced into the backbone, which results in p-doping or n-doping respectively. Figure 1-3 shows the chemical structures of several CPs commonly used.

![Chemical structures of conducting polymers](image)

**Figure 1-3.** Chemical structures of conducting polymers: polyacetylene is the first reported CP; polypyrrole, polythiophene, poly(3,4-ethylenedioxythiophene) and polyaniline are commonly used in biomedical applications.

CPs can be synthesized either chemically or electrochemically, with each having their advantages and disadvantages. Chemical polymerization enables large-scale production and more options to modify CP backbone covalently, but the synthesis is more complicated and not able to control the thickness. In contrast, electrochemical deposition is preferred when the control of film thickness is required, by simply adjusting potential and time of polymerization.

42 In contrast, electrochemical deposition is preferred when the control of film thickness is required, by simply adjusting potential and time of polymerization.43
One other advantage of electrochemical method is the straightforward entrapment of anionic molecules during polymerization. In the framework of this study, CP films were electrochemically deposited on an electrode; accordingly, all the information given below is referred to this method. Electrochemical polymerization is performed using a three-electrode-cell, which contains a monomer, appropriate solvent, and dopant. Current is passed through the solution and electro-deposition occurs at the positively-charged working electrode. Monomers in the vicinity of the electrode surface undergo oxidation to form radical cations that react with other monomers or other radical cations, forming polymer chains on the electrode surface. Upon doping, a CP system with a zero net charge, due to the association of counter anions with the charged CP backbone, is produced. Figure 1-4 shows the mechanism of electrochemical polymerization of PPy as an example. This process introduces the charge carriers in the form of charged polarons (i.e. radical ions) or bipolarons (i.e. dications or dianions), into the polymer. The attraction of electrons in one repeat-unit to the nuclei in neighboring units yields charge mobility along the chains. The ordered movement of these charge carriers along the conjugated CP backbone produces electrical conductivity.42

\[
\begin{align*}
\text{PPy} & \quad \text{(1)} \\
\text{PPy}^+ & \quad \text{(2)} \\
\text{PPy}^{2+} & \quad \text{(3)} \\
\text{PPy}^{2+} & \quad \text{(4)} \\
\text{PPy}^{3+} & \quad \text{(5)} \\
\text{PPy}^{3+} & \quad \text{(6)} \\
\text{PPy}^{4+} & \quad \text{(7)} \\
\text{PPy}^{n+} & \quad \text{(8)} \\
\end{align*}
\]

**Figure 1-4.** Mechanisms of electrochemical polymerization for PPy42,44

### 1.3.2. Immobilization of biomaterials into CPs

In addition to the applications listed above, CPs offer many advantages in the biological fields as well; most CPs present biocompatibility, an ability to reversibly entrap and release biological molecules (i.e. doping and dedoping), and an ability to transfer charges by a biochemical reaction.42 These unique characteristics are useful in many biomedical applications,
such as biosensors, tissue-engineering scaffolds, neural probes, drug-delivery devices, and bio-actuators. In this section, studies that are related to the incorporation of biomaterials onto PPy and PEDOT are reviewed.

Biomaterials can be immobilized to CPs film by physical adsorption or entrapment, covalent binding, crosslinking, or a combination of all these techniques. Physical adsorption is the simplest method for immobilization, in which the biomolecules are adsorbed at the polymer interface, thanks to static interactions between the polycationic surface of the oxidized polymer and the negative charge of enzyme. Among CPs, PPy is extensively used for immobilization of biomolecules and living cells as well, due to the biocompatibility. Since 1989, glucose was detected in a wide concentration range of 2.5-30 mM by the adsorption of glucose oxidase (GOD) onto an electropolymerized PPy film. For cholesterol sensing, cholesterol oxidase and a mediator have been incorporated into PPy films. Despite of the simplicity, the weak non-covalent forces involved in the oxidase adsorption often lead to unstable immobilization, which decreased the lifetime of the device.

Another technique alternative to adsorption is the entrapment of a biomaterial during electropolymerization. In an early study, Foulds and Lowe described a method for incorporating GOD into PPy films in a manner that was capable of producing glucose-sensing electrodes in a one-step process. These electrodes responded rapidly, reaching a steady state within 20-40 s. It was reported first time that the direct electron transfer between PPy-entrapped quinohemoprotein-alcohol-dehydrogenase from *Gluconobacter sp.* and a platinum electrode took place via the conducting-polymer network. The cooperative action of the enzyme-integrated prosthetic groups - pyrroloquinoline-quinone and hemes was supposed to allow the electron-transfer pathway from the enzyme's active site to the CPs backbone. Entrapment methods have also been used for immobilization of antibodies and DNA. The advantage of covalent-modification over physical adsorption and entrapment is the strong binding between the biomaterials and the polymer matrix. An electrochemical sensor for DNA has been developed based on PPy functionalized with oligonucleotide (ODN). The electrochemical response of this ODN-functionalized PPy electrode has then been analyzed in various aqueous media, containing either complementary or noncomplementary ODN targets. Upon the addition of complementary ODN targets, a significant modification in cyclic voltammetric response was observed, indicating the specific hybridization between the polypyrrole-grafted ODN probe and its complementary ODN target. The sensor performed a detection limit of $10^{-11}$ mol.
covalent-binding of various enzymes including quinohemoprotein alcohol dehydrogenase,\textsuperscript{54} and GOD\textsuperscript{55} to PPy functionalized for detection of alcohol and glucose respectively was also reported.

In comparison with other CPs, PEDOT appears as a more friendly substrate for immobilizing biomaterials because of the more rapid formation in neutral pH region as well as the excellent stability and biocompatibility in ambient condition.

A significant number of studies on the interaction between PEDOT and neural cells have been conducted. Richardson-Burns et al. examined the electrochemical polymerization of PEDOT directly within a neural cell and found the formation of a conductive network that was integrated within the tissue and enabled uniquely intimate and sensitive contacts between the electrode and the plasma membranes of the neural cells. In addition to the brain tissue, functional PEDOT networks could be polymerized within a variety of other tissues.\textsuperscript{56} In another study, in which they made an attempt to create a template of neural cell imprinted PEDOT, it was found that the brain tissue embedding in the PEDOT remained alive for almost 1 week, while the PEDOT-surrounded neurons started to die after 24-72 h of an initial insult.\textsuperscript{57} Other investigations on bioelectrochemical control of a neuron development on a PEDOT/polystyrene sulfonate (PSS) modified electrode,\textsuperscript{58} and the development of PEDOT-biomolecule composites for neural electrodes\textsuperscript{59} have been reported as well.

Türkarslan and co-workers entrapped cholesterol oxidase (ChOx)\textsuperscript{60} and alcohol oxidase (AlcOx)\textsuperscript{61} into PPy and PEDOT via the electrochemical polymerization of the solution consisting of monomer, the protein, and sodium dodecyl sulfate (SDS) as supporting electrolyte and surfactant. They found that enzyme modified PEDOT electrodes showed higher affinity towards the targeted substrates for both cholesterol and alcohol. An amperometric enzyme sensor for detection of phenolic compounds and herbicides was fabricated using tyrosinase, also called polyphenol oxidase. The enzyme was entrapped into a PEDOT film electrochemically generated on a glassy carbon electrode. The detection limits for phenol and herbicides (atrazine and diuron) were ranging from 5 to 500 nM and 0.5 to 1 mg L\textsuperscript{-1} respectively.\textsuperscript{62}

In recent years, virus became a promising recognition material for electrical conductivity-based biosensing due to the densely packed receptors in their protein coats, which can essentially recognize any analyte via binding. Grafting M13 bacteriophage into an array of PEDOT nanowires generated hybrids of CPs and viruses.\textsuperscript{63} The electrochemical impedance of virus-PEDOT films increased upon exposure to an antibody that selectively binds to the M13 coat peptide. Exposure to the antibody caused a shift in both real (ZRE) and imaginary (ZIM)
impedance components across a broad range of frequencies from 50 Hz to 10 kHz. The electrical conductivity of the virus-PEDOT film, measured using conductive tip AFM, decreased linearly with virus loading, from 270 μS cm\(^{-1}\) for pure PEDOT films to 50 μS cm\(^{-1}\) for films containing 100 μM virus.
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CHAPTER II
Observation of Bacterial Activity Using Polypyrrole Films
Doped with Bacteria

2.1. Introduction

In addition to a wide range of electronic applications, conducting polymers (CPs) have been utilized as entrapping materials in the field of biosensors.1−8 Biomaterials, such as enzymes and DNA, can be immobilized on substrates through adsorption, entrapment, covalent binding, cross-linking, or a combination of all these techniques.9−12 Entrapment of viable microbes has also been studied, using matrices, such as calcium alginate, agar, cellulose, and polyamide. Whole-cell biosensors is one application of such immobilization techniques.12−14 CPs are different from these materials in that they can automatically entrap microbes during polymerization.

In spite of the usefulness of CPs as entrapping materials, only a limited number of studies using CPs as an inclusion matrix for bacteria have appeared in the literature. *Gluconobacter oxydans* and *Pseudomonas fluorescens* cells were immobilized on graphite electrodes modified with CPs such as polypyrrrole (PPy) and polythiophene for the detection of glucose.15−17 A self-doped PPy was synthesized from a pyrrole-alginate monomer, which was polymerized on an electrode after application of a CaCl$_2$ solution.18,19 Amperometric biosensors coated with a regular alginate gel and the synthesized PPy-alginate matrix capturing algal cells of *Chlorella vulgaris* have been comparatively studied.19 Using polystyrene sulfonate (PSS), aniline monomer, and lyophilized bacterial biomass (*Brevibacterium ammoniagenes*), a polyaniline film entrapping the bacterial cells was deposited at a pH of 4 between twin Pt-wire electrodes for use in urea determination.20 Immobilizing techniques based on polythiophene have also been reported.17, 21

However, the above studies have used CP films either as adsorption platforms, over which microbes are spread, or as inclusion matrices, and none of these studies has explicitly used the valuable property of doping that CPs possess. Previously, the direct insertion of various bacteria into the PPy films,22 which is made possible by the negative-charge on bacterial cell walls has been reported.23 In this current study, characteristics of the polymer films, as well as the activity of bacteria doped in the polymer films were analyzed. Here, the following issues are discussed:
(1) the film structure prepared under various pH, (2) the viability of bacteria doped in the polymer film, as well as their tolerance for attachment to electrode materials, (3) the activity of bacteria involved in the film, and (4) prospects for further application.

2.2. Experimental

2.2.1. Chemicals and materials

Monomer, 3,4-ethylenedioxythiophene (EDOT) was purchased from Sigma-Aldrich Co. Pyrrole, potassium dihydrogen phosphate (KH$_2$PO$_4$), disodium hydrogen phosphate (Na$_2$HPO$_4$), and sodium chloride (NaCl) were obtained from Wako Pure Chemical Industries (Japan). Alginic acid was purchased from MP Biomedicals (France). Pyrrole was distilled before use, but all the other reagents were of pure analytical grade and used as received.

Nutrient Broth (NB) and tryptone soya broth (SCD broth) were obtained from Eiken Chemicals (Japan). The bacterial viability kit, LIVE/DEAD BacLight L7007, was purchased from Molecular Probes. Bacterial samples of *Pseudomonas aeruginosa*, *Escherichia coli* (O Rough:H48), *Acinetobacter calcoaceticus*, *Serratia marcescens* and *Bacillus subtilis* were acquired from the Biological Resource Center (NBRC, Japan) and *Shewanella oneidensis* MR-1 was obtained from American Type Culture Collection (ATCC). Ultrapure water (resistance >18 MΩ) was used throughout this work.

2.2.2. Bacterial cultivation

*P. aeruginosa*, *E. coli*, *S.oneidensis*, *A.calcoaceticus* and *S.marcescens* were cultured in the NB broth, while *B.subtilis* was grown in the SCD broth overnight at 30°C. The bacterial suspensions were centrifuged at 2000 g for 10 min and the supernatant was discarded. The bacterial pellet was then resuspended in 0.85% NaCl (saline). This isolation procedure was repeated twice to obtain a purified bacterial target.

2.2.3. Electrochemical experiments

All the microbial experiments were performed under strictly sterile conditions. All electrochemical experiments were performed with a potentiostat (model 842B, ALS) equipped with quartz microbalance (SEIKO EG&G.).
**a/ Preparation of bacteria-doped PPy**

A Ag | AgCl | saturated KCl || electrode and platinum wire electrode were served as reference and counter electrodes. Unless otherwise noted, a film of PPy doped with *P. aeruginosa* (PPy/*P. aeruginosa*) was prepared as follows: pyrrole (5 µmol) and 0.50 mL of a phosphate buffer (pH 2.5 – 5.3) were added to the cell precipitate obtained above. This dispersion had a pyrrole concentration of 10 mM and a *P. aeruginosa* density of 1.5×10⁹ CFU mL⁻¹. When to see the effect of alginate doping in the PPy film, a polymerization mixture with 0.33% alginate was prepared. These dispersions were oxidized at 0.98 V against the Ag|AgCl electrode for 300 s to deposit a PPy film on a quartz-crystal-microbalance (QCM) gold electrode (Seiko EG&G; surface area, 0.2 cm²), which had been pretreated by soft plasma etching (Meiwa Fosis, SEDE-GE) for 30 s. The PPy film thus deposited gave a film thickness of a few micrometers. Films doped with other bacteria were prepared in the same manners. A strip of a glassy carbon electrode was polished with fine aluminium powder, and PPy films were prepared in the same way.

**b/ Preparation of bacteria-doped PEDOT**

A sheet of indium tin oxide (ITO)-coated glass (resistance: 10 Ω cm⁻²) was cut into a 0.5 x 1.3-cm strip, sonicated in ethanol for 30 min and then coated with an adhesive polypropylene tape (Scotch® Filament Tape 898), which has a hole (diameter, 4 mm) for use as the working electrode. The Ag|AgCl electrode, and a platinum mesh (0.7×1.5 cm²) were employed as the reference, and counter electrodes, respectively. The electrodes were placed in a glass cell, which contained 2 mL of aqueous solution consisting of 10 mM EDOT and purified bacteria (2.6×10⁷ cells mL⁻¹) in a pH 5.3 phosphate buffer. A constant potential of 1.05 V was applied for 100 s to deposit a PEDOT film on the ITO electrode. Freshly polymerized PEDOT/bacteria films were then rinsed with an ample amount of water and immediately subjected to microscopic analyses.

**c/ Thin-layer-cell voltammetry**

To monitor the bacterial activity a thin-layer electrochemical cell was employed. Polypyrrole films doped with *E. coli* were deposited on ITO glass (2.6×7.6 cm²), which has a working electrode area of 4 mm in diameter described in the same way as above. After the PPy deposition, the electrode was rinsed with water and a pH 7.0 phosphate buffer 700 µL was applied only on this area. Afterwards, a piece of filter paper (No. 1, diameter 55 mm, Advantec. Co., Japan), which was folded in half, was placed on the ITO glass strip. Another ITO glass,
used as the counter electrode, was placed on the filter paper, which stuck the paper ca. 5 mm out
the glass sandwich. This cell assembly was fixed and bound with an adhesive Teflon tape. A
reference electrode tip (ca 1 mm in diameter) was inserted in between the two layers of the filter
paper sticking out to make a stable electrical contact. Between voltammetric examinations, the
cell was disconnected from the potentiostat to minimize the oxygen consumption at the working
electrode and its generation at the counter electrodes, both of which occurred in parallel to
oxygen consumption based on the bacteria activity. All the thin layer cell experiments were
performed at 37°C in a thermostated Faraday cage.

2.2.4. Microscopic observation

The bacteria doped CPs film was stained with fluorescence pigments, SYTO9 and
propidium iodide (PI), according to the manufacturer’s instructions for the Bacterial Viability
Kit (Molecular Probes). Bacteria immobilized in the polymer films were observed by
fluorescent microscope (BX51, Olympus Co.; Japan). The surfaces of the polymer films were
also imaged by scanning electron microscopy (SEM; TM-1000, Hitachi; Japan).

2.3. Results and Discussion

PPy is one of the most commonly used CPs and is prepared by the oxidation of pyrrole
monomer (PyH₂) as following:

\[
m \cdot \text{nPpy} \cdot \text{H}_2 + mX^- + m(2n+1)e^- \rightarrow (-\text{Py}_\text{n}^-)_{\text{m}} \cdot (X^-)_{\text{m}} + 2m \cdot n\text{H}^+
\]

where X⁻ is the dopant, which is taken up into the polymer upon polymerization. The
polypyrrole salt, \((-\text{Py}_\text{n}^-)_{\text{m}} \cdot (X^-)_{\text{m}}\), is denoted hereafter as PPy/X⁻. The automatic injection of the
anionic molecule is a key feature of CPs with respect to their applications, in which the insertion
of the functional substances, such as bacteria, is expected to occur regardless of their bulkiness,
as long as these substances carry negative charges.

2.3.1. Immobilization of bacterial cell

Scheme 1 illustrates bacterial immobilization techniques using CPs discussed in this study
as well as those reported in the literature. Panel (A) shows a simple fixation method in which a
bacterial suspension is applied on a PPy film. It was pointed out a decade ago that cell growth
could be modulated by controlling the redox states of a PPy film. To entrap bacteria in PPy-
based matrices, polymer films prepared from a self-doping pyrrole-alginate monomers have
been reported to introduce gel structures in the presence of Ca²⁺ to enhance biocompatibility and
permeability for the PPy films (B). The pKa values of 3.4 and 3.7, reported for mannuronic and guluronic acids found in alginic acid, indicate that the polypyrrole-alginate is self-doped in the pH range above 4.18,19

**Scheme 2-1.** Several bacteria immobilization techniques referenced in this study

(A) Adsorption of bacteria on a PPy film, (B) a self-doped conducting polymer film entrapping bacteria prepared from the pyrrole-alginate monomer, Py-Alg; (C) a PPy film doped with bacteria and buffer components, (D) a PPy film doped with bacteria, alginate and buffer components.

Alternatively, a more classical and direct approach to immobilize bacteria into polypyrrole films have been adopted (C and D). Polypyrrole was doped with anions during oxidative polymerization as shown in the equation (1), and the insertion of bacteria was found possible by the presence of outer cell membranes that abundantly contained negatively charged lipids, such as lipopolysaccharides found for Gram-negative bacteria. Actually, zeta potentials (ζ-potentials) of –34 and –28 mV were observed for *P. aeruginosa* and *A. calcoaceticus*, respectively, to give experimental evidence of the dominance of the anionic residue on the cell surfaces. Gram-positive bacteria, which do not possess the lipopolysaccharides on the outer cell walls, were also doped into the CP films. For instance, *B. subtilis* have pKa values of 4.8, 6.9, and 9.4, and 16, which likely correspond to carboxyl, phosphate and hydroxyl sites, respectively, and the former two sites are accordingly dissociated in the neutral pH region to provide the negative charge on the surface. Metal ion sorption experiments on *B. subtilis* evidenced that anionic groups also dominate the surface in the neutral pH region,25,26 and the observed zeta potential was actually negative (-34 mV).27
2.3.2. Film structures of inserted bacteria

SEM images of five different bacilliform bacteria used in this study are shown in Fig. 2-1, in which *A.calcoaceticus*, *S.marcescens*, *E.coli*, and *P.aeruginosa* are Gram-negative bacteria, and *B.subtilis* is Gram-positive bacterium. Sizes of bacteria are in range of 0.56 - 0.81 µm in width and 0.88 - 1.72 µm in length.

![SEM images of five different bacilliform bacteria](image)

**Figure 2-1.** SEM images of (A)*Acinetobacter calcoaceticus*, (B)*Serratia marcescens*,(C)*Bacillus subtilis*, (D)*Escherichia coli*, and (E)*Pseudomonas aeruginosa*

![SEM images of PPY/P. aeruginosa](image)

**Figure 2-2.** SEM images of PPY/*P. aeruginosa* in the course of polymerization at pH 2.5. Polymerization period, (A) 30 s, (B) 40 s, and (C) 60 s.
Figure 2-2 (A) through (C) shows the SEM images of *P. aeruginosa* during PPy polymerization at pH 2.5. The bacteria were immobilized in a self-standing fashion even at the very first moment of the polymerization (30 s), at which a polymer film was scarcely seen. More bacteria were fixed on the surface at 40 s, and their bottom parts were clearly embedded in the film within 60 s. Interestingly, all five bacteria used in this study were vertically and quite densely inserted into PPy films at low pH as shown in Figure 2-3.

![SEM images of PPy/A. calcoaceticus, PPy/S. marcescens, PPy/B. subtilis, PPy/E. coli, and PPy/P. aeruginosa.](image)

**Figure 2-3.** SEM images of (A) PPy/A. calcoaceticus, (B) PPy/S. marcescens, (C) PPy/B. subtilis, (D) PPy/E. coli, and (E) PPy/P. aeruginosa. The films were prepared by polymerising 10 mM pyrrole for 300 s at pH 2.5 on QCM gold electrodes.

In contrast to the results obtained at pH 2.5, bacteria were not vertically inserted at pH 5.3 even for a polymerization of 900 s and were fixed almost horizontally (Fig. 2-4). This structure is probably due to a smaller initial polymerization rate of PPy in the neutral pH region. Figure
2-4 (A, B) shows that every *E. coli* was horizontally doped, although their doping levels were different at observed locations. PEDOT grows much faster than PPy in neutral pH (note the difference in the polymerization time), and panel (C) indicates that the polymerization rate actually altered the injection structure.

![SEM images of (A, B) PPy/*E. coli* observed at different locations, and (C) PEDOT/*E. coli*.](image)

**Figure 2-4.** SEM images of (A, B) PPy/*E. coli* observed at different locations, and (C) PEDOT/*E. coli*. Polymerization conditions: (A, B) at pH 5.3 and at 0.98 V for 900 s; (C) at pH 5.3 and at 1.05 V for 100 s. Film thickness; (A, B) ~0.8 µm, and (C) ~0.4 µm.

![Viability of entrapped *P. aeruginosa* observed with a fluorescent microscope after staining a PPy film with SYTO9/PI fluorochrome reagents.](image)

**Figure 2-5.** Viability of entrapped *P. aeruginosa* observed with a fluorescent microscope after staining a PPy film with SYTO9/PI fluorochrome reagents; the images were observed for (A) all the cells (SYTO 9 and PI emissions; green) and for (B) the dead cells (PI emission; red). The PPy film was prepared by polymerizing 0.010 M pyrrole at pH 5.3 for 900 s with 66 ppm alginate. The fluorochromes were excited under illumination of (A) 460–490 and (B) 510-550 nm wavelengths, and observed through high-pass optical filters with cut-off wavelengths of (A) 520 and (B) 590 nm.
Similar evidence revealing the importance of the polymerization rate is shown in the fluorescent microscopic images of PPy/(alginate, \textit{P. aeruginosa}) films stained by both SYTO 9 and PI, which were added after preparation of the film (Fig. 2-5). The images reveal that the PPy film doped with alginate gives circular radiants, suggesting that most of the bacteria were vertically inserted in the film even at a polymerization pH of 5.3, and that the film was more quickly deposited by the acceleration with alginate. It has been reported that polyanionic dopants, such as polystyrene sulfonate (PSS), accelerate the rate of polymerization, and a PSS-polyaniline film entrapping the bacterial cells was deposited on Pt-wire electrodes at pH as high as 4.\textsuperscript{20}

\textbf{2.3.3. Viability of bacteria in conducting polymer films under different polymerization conditions}

Viability of bacteria, \(\%V\), was evaluated with a fluorescent microscope using the fluorescent dyes; an example of such analysis is given in Fig. 2-5, in which the number of the red cells (B) were counted as the dead cells, \(N_D\), and the number of the green and yellow cells (A) were counted as the total number, \(N_T\), to define \(\%V\) as \((N_T - N_D)/N_T\). Viabilities observed in different polymerization conditions are listed in Table 2-1. The fact that \textit{P. aeruginosa} gave a low viability of <20\% after polymerization at pH 2.5 discouraged the fancy of studying their electrochemistry at this pH in more detail. Accordingly, the films have been prepared at a higher pH of 5.3 at the cost of slower film growth rates to discuss their characteristics in the following sections. \textit{S. oneidensis} and \textit{E. coli} showed viability and stability much higher than \textit{P. aeruginosa}, resulting in little difference in the way the films had been polymerized. By this reason, the effect of polymerization conditions on bacterial viability during immobilization process at pH 5.3 has been focused mainly on \textit{P.aeruginosa}.

The number of bacterial cells on the electrode surface augmented with an increase in the electrolysis period as expected (Fig. 2-6). The polymerization time of zero in this figure was for the bare gold electrode, which was soaked into the bacterial dispersion for 30 min and then rinsed with an ample amount of water, followed by the fluorescent microscopic analysis. The electrode few retained the bacteria on its surface, and the surface density was evaluated to be about 1/1000 as many as that for PPy/\textit{P. aeruginosa} (see also Table 2-1). Although the viability of the adsorbed bacteria was 30\%, the small surface density would not be served to practical applications. Besides, the polarization of this electrode for 900 s at 0.98 V made their viability nil (◇), which contrasts with the value of 38\% for doped bacteria, demonstrating that the PPy
matrix protected the bacteria from the voltage stimulus more efficiently than the bare metallic surface. In Table 2-1, the observed viability was corrected for the effect of dead cells, since the cultured *P. aeruginosa* contained ~20% dead cells, which were inserted into the PPy films as well as the live ones. After this correction, the viability for PPy/*P. aeruginosa* was improved by ~10%.

![Figure 2-6](image)

**Figure 2-6.** Effect of polymerization time on (●) the surface density and (◆) observed viability of *P. aeruginosa*. Polymerization was conducted from a solution containing 10 mM pyrrole and *P. aeruginosa* at pH 5.3 at 0.98 V. The symbol (◇) shows the viability of the bacteria adsorbed on a bare Au electrode 900 s after an application of 0.98 V.

Figure 2-7 shows the effect of alginate in the PPy matrix. Although the number of immobilized *P. aeruginosa* in the film decreased with an increasing concentration of alginate in the polymerization mixture due to the doping event competing with each other, there was an appreciable change in the viability at an alginate concentration of 350 ppm to reach 70%. Alginate forms hydrogels by cross-linking of the polymer strands with calcium ion, leading to a three dimensional network (Scheme 2-1 B). It is interesting that alginate without Ca$^{2+}$ ions showed a substantial effect on improving the viability without constructing such gel structures, leading to the increase in the corrected viability up to 89% (Table 2-1).
Figure 2-7. Effect of alginate on the surface bacterial density and observed viability; PPy/P. aeruginosa was polymerized from a solution containing 10 mM pyrrole, the bacteria and alginate. The accuracy of the viability estimated from the standard deviation (n=4) is ca. ±10 % for each measurement.

The effects of different electrode materials on the viability have also been studied: A glassy carbon electrode few retained P. aeruginosa by adsorption, and PPy/P. aeruginosa, deposited on carbon appreciably improved the corrected viability (62%), which was higher than that for the gold electrode (48%). Further, dual coating has been examined, in which PPy undercoating was prepared from the mixture of pyrrole monomer and buffer on the Au electrode, expecting that a PPy surface is more biocompatible than the Au surface. The viability of the bacteria doped on this layer was improved more than that for the PPy/P. aeruginosa prepared on the Au electrode. A different polymer, PEDOT, was studied to show a similar viability for PPy.

E. coli exhibited much higher viability and stability than P. aeruginosa and was even so without a PPy matrix. Different from P. aeruginosa, E. coli was quite densely adsorbed on the glassy carbon surface (Table 2-1), but was prone to cohere and build up into layers, seen as scattered islands under microscopic observation. In contrast to the adsorbed surface, PPy/E. coli gave a well-dispersed surface, and the difference in these images was numerically expressed in terms of the standard deviation of 5.7 and 1.3 cells cm\(^{-2}\) for adsorbed E. coli and PPy/E. coli on glassy carbon, respectively (number of viewed fields, 10, Table 2-1). In contrast to glassy carbon, an ITO surface few retained E. coli with a poor viability, making PPy as an excellent immobilization material on the glass.
Table 2-1. Polymerization conditions affecting the viability of *P. aeruginosa*, *E. coli* and *S. oneidensis* in the PPy and PEDOT films a)

<table>
<thead>
<tr>
<th>Film</th>
<th>Base electrode material</th>
<th>Polymerization condition b)</th>
<th>Surface density / 10⁶ cells cm⁻²</th>
<th>%V₀</th>
<th>%Vₜₜₜ</th>
<th>%Vₜₜₜ c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Period/s pH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ads</td>
<td>Au</td>
<td>-</td>
<td>0.002</td>
<td>72</td>
<td>30</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>GC</td>
<td>-</td>
<td>0.005</td>
<td>72</td>
<td>26</td>
<td>36</td>
</tr>
<tr>
<td>PPy/Pa</td>
<td>Au</td>
<td>900</td>
<td>5.3</td>
<td>3.3</td>
<td>79</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Carbon d)</td>
<td>900</td>
<td>5.3</td>
<td>2.9</td>
<td>68</td>
<td>42</td>
</tr>
<tr>
<td>PPy/Pa + 330 ppm alginate</td>
<td>Au</td>
<td>900</td>
<td>5.3</td>
<td>2.3</td>
<td>79</td>
<td>70</td>
</tr>
<tr>
<td>PPy/Pa on PPY/phosphate</td>
<td>Au</td>
<td>900</td>
<td>5.3</td>
<td>2.5</td>
<td>68</td>
<td>39</td>
</tr>
<tr>
<td>PEDOT/Pa</td>
<td>Au</td>
<td>180</td>
<td>2.6</td>
<td>2.1</td>
<td>88</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>ITO</td>
<td>180</td>
<td>5.3</td>
<td>2.8</td>
<td>88</td>
<td>48</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ads</td>
<td>GC</td>
<td>-</td>
<td>11.2±5.7</td>
<td>97</td>
<td>92</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>ITO</td>
<td>-</td>
<td>&lt;0.001</td>
<td>97</td>
<td>67</td>
<td>69</td>
</tr>
<tr>
<td>PPy/Ec</td>
<td>GC</td>
<td>900</td>
<td>5.3</td>
<td>7.8±1.3</td>
<td>97</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>ITO</td>
<td>900</td>
<td>5.3</td>
<td>13.2±2.0</td>
<td>97</td>
<td>95</td>
</tr>
<tr>
<td>PPy/Ec + 330 ppm alginate</td>
<td>ITO</td>
<td>900</td>
<td>5.3</td>
<td>5.7</td>
<td>97</td>
<td>93</td>
</tr>
<tr>
<td><em>S. oneidensis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ads e)</td>
<td>ITO</td>
<td>-</td>
<td>0.086±0.06</td>
<td>96</td>
<td>95</td>
<td>99</td>
</tr>
<tr>
<td>PPy/So</td>
<td>ITO</td>
<td>600</td>
<td>5.3</td>
<td>1.2±0.8</td>
<td>96</td>
<td>84</td>
</tr>
<tr>
<td>PPy/So</td>
<td>ITO</td>
<td>900</td>
<td>5.3</td>
<td>14.6±1.9</td>
<td>96</td>
<td>50</td>
</tr>
</tbody>
</table>

a) Abbreviations: Pa = *P. aeruginosa*, Ec = *E. coli*, So = *S. oneidensis*, ads = adsorbed bacteria, GC = glassy carbon.

b) Polymerization was conducted at +0.98 V vs. Ag/AgCl.

c) The viability corrected for the immobilization of the dead cells, %Vₜₜₜ, is expressed in terms of:

\[ \%V_{\text{corr}} = \frac{\%V_{\text{obs}}}{\%V_{\text{obs}}} \times 100, \]

where %Vₜₜₜ and %V₀ are the experimentally obtained per-cent viability, and the per-cent viability of the cultured bacteria, respectively.

d) Carbon film deposited on the QCM gold electrode.

e) There were two structures for the adsorption; the values listed were for well-separated bacteria. See text in detail.
Similar to *E. coli*, *S. oneidensis* were not well distributed as adsorbed on ITO glass. The adsorbed bacteria composed of mixed substructures, with some cohered like island (density, $1.3 \times 10^7$ cells cm$^{-2}$; %$V_{obs}$, 32%), but some separated (density, $8.6 \times 10^4$ cells cm$^{-2}$; %$V_{obs}$, 95%), showing quite a wide range of distributions in both density and viability. However, PPy/S. oneidensis gave a uniform and dense structure for the immobilized bacteria.

2.3.4. Voltammetric responses of PPy/E. coli

As listed in Table 2-1, *E. coli* had much higher viability than *P. aeruginosa*. The %$V_{corr}$ value of PPy films for *E. coli* was much higher (96-99%) than that for *P. aeruginosa* (48-62%). They were also viable after voltammetric experiments; no appreciable changes in the viability for PPy/E. coli were observed before and after voltammetric examination discussed in this section. Therefore, the voltammetric behaviour of *E. coli* rather than *P. aeruginosa* has been studied due to its stability.

![Voltammetric observation of E. coli activity recorded in an aerobic condition using the thin layer electrolysis cell at a scan rate of 10 mV s$^{-1}$ and at 37°C.](image)

Figure 2-8. Voltammetric observation of *E. coli* activity recorded in an aerobic condition using the thin layer electrolysis cell at a scan rate of 10 mV s$^{-1}$ and at 37°C. (A) cyclic voltammograms (——) in the first pH 7.0 phosphate buffer, (-----) in 10 mM glucose dissolved in the pH 7.0 phosphate buffer, and (——) in the replaced pH 7.0 phosphate buffer; all were recorded 30 min after cell packing. (B) Oxygen reduction currents at -0.8 V; (PB1) in the first buffer solution, (Glu (0 min)) in the 10 mM glucose solution observed immediately after cell packing, (Glu (30 min)) in the same solution observed 30 min after packing, and (PB2) in the replaced buffer solution at the (○) PPy/E. coli and (△) PPy/phosphate electrodes.

Figure 2-8 shows results of voltammetric experiments using the PPy/E. coli films assembled in the thin layer electrochemical cell (*see* the experimental section), under an aerobic condition. This cell enabled oxygen consumption monitoring based on bacteria respiratory in a highly efficient manner by limiting the volume of the electrolysis solution. Voltammograms of *E. coli* recorded in an anaerobic condition were featureless, suggesting that redox species were
not appreciably expressed on the cell membrane in contrast to redox bacteria often used in biological fuel cells, such as *Geobacter sulfurreducens*. An air-saturated phosphate buffer showed oxygen reduction at potentials below -0.3 V, and oxygen consumption by bacteria was clearly traced on the addition of glucose in the buffer, as seen in Figure 2-8 (A). Replacing the buffer to the fresh phosphate buffer without glucose after this experiment fully recovered the oxygen peak, indicating that the decrease of the current was not attributed to fouling of the electrode surface but was due to the consumption of dissolved oxygen by bacteria (B).

![Figure 2-9](image.png)

**Figure 2-9.** Oxygen consumption in the thin layer cell at 37°C: (A) The current on the PPy/*E. coli* ITO electrode at -0.8 V and at 10 mV s⁻¹ against the glucose concentration; (○) the first buffer and (●) the buffer replaced after the experiment for 50 mM glucose; waiting time, 30 min. (B) Time profiles of the currents at -0.8 V of 20 mM glucose at 50 mV s⁻¹ (scan range, 0.0 – -0.8 V) for (●) the PPy/*E. coli* and (▲) PPy/phosphate ITO electrodes.

The rate of the oxygen consumption linearly increased with an increase of the glucose concentration (Fig. 2-9 A) up to about 20 mM, and again the electrode fouling was not found after the experiments. Figure 2-9 B shows time profiles of the oxygen consumption, which were continuously recorded without disassembling the cell; here, a higher scanning rate and narrower scan range were adapted for shorter observation time. Firstly, the oxygen consumption at the working electrode was monitored using an electrode without bacteria (▲); the electrical connection of the cell to the potentiostat was switched off after each scan to minimize the electrochemical oxygen consumption, which could occur in parallel to the bacterial respiratory. Although a small decrease in the current without *E. coli* (▲) was observed due to this effect, much larger current decreases were detected for the bacteria-loaded film (●), indicating that about 2/3 of oxygen dissolved in the filter paper were consumed by the bacteria in an hour at 37°C. It is noteworthy that these voltammetric procedures function as a simple but powerful analyzing tool to monitor bacterial activity.
2.4. Conclusion

The immobilization state of bacteria in different matrix and at different pH indicated that rate of polymerization alters the injection structure. Compared to various materials, including gold QCM electrode, glassy carbon, and ITO glass CPs appeared as the best substrate for entrapping bacteria with respect to their density, viability, and distribution. The respiratory activity of bacteria doped on the polymer film showed clear responses with the addition of glucose as nutrient, promising a simple but powerful analyzing tool to monitor bacterial activity.
REFERENCES


CHAPTER III

Viability Analysis of Bacteria Doped on Poly(3,4-ethylenedioxythiophene) (PEDOT) Films through Electrochemistry

3.1. Introduction

During the second half of the 1980s, scientists at the Bayer AG research laboratories in Germany developed a new polythiophene derivative, poly(3,4-ethylenedioxythiophene) (PEDOT), which was initially found to be an insoluble polymer, yet exhibited some very interesting properties. In addition to a very high conductivity (ca. 300 S cm⁻¹), PEDOT was found to be almost transparent in thin, oxidized films and showed a very high stability in the oxidized state.¹

In previous chapter, the immobilization state of various bacteria in PPy film was clearly defined, either horizontally doped or self-standing fashion; the respiration activity of bacteria was also investigated. In this chapter, the viability of bacteria during and after entrapment into PEDOT matrix will be discussed. Furthermore, the electrochemical behavior of the PEDOT films prepared under different conditions of polymerization potential and time is evaluated in relation to the viability of doped bacteria.

As compared to other CPs, PEDOT is easily formed in the neutral pH region, which provides a mild condition for biomaterial. Recent findings have demonstrated the biocompatibility of PEDOT. Türkarslan and co-workers reported that the enzymes, cholesterol oxidase and alcohol oxidase were entrapped in PEDOT matrices and successfully detected the respective targets - cholesterol and alcohol.²³ Another group prepared a composite of PEDOT and M13 bacteriophage, a bacteria infected virus through electrochemical polymerization, in which detecting of antibody selectively bound to M13 virus is impedance-based biosensing.⁴⁵ The observation in my study, which employed the same procedure of entrapment into PEDOT, through a single-step electrochemical oxidation showed a 98% of bacterial living rate after immobilization; this reinforces the excellent biocompatibility of PEDOT. Similar to the case of PPy, along with anionic molecules of supporting electrolytes, bacteria acted as dopants during polymerization.
3.2. Experimental

3.2.1. Chemicals and materials

All the reagents were of analytical grade and used without further purification. Monomer, 3,4-ethylene dioxythiophene (EDOT) was purchased from Sigma-Aldrich Co. Potassium dihydrogen phosphate (KH₂PO₄), disodium hydrogen phosphate (Na₂HPO₄), and sodium chloride (NaCl) were obtained from Wako Pure Chemical Industries (Japan). A nutrient broth was purchased from Eiken Chemical (Japan). The bacterial viability kit, LIVE/DEAD BacLight L7007, was purchased from Molecular Probes. Bacterial samples of Pseudomonas aeruginosa and Escherichia coli were acquired from the Biological Resource Center (NBRC, Japan), and Shewanella oneidensis was obtained from American Type Culture Collection (ATCC). Ultrapure water (resistance >18 MΩ) was used throughout this work.

3.2.2 Bacterial cultivation

P. aeruginosa, E. coli, and S. oneidensis were cultured in nutrient broth overnight at 30°C. The bacterial suspensions were centrifuged at 2000 ×g for 10 min and the supernatant was discarded. The bacterial pellet was then resuspended in 0.85% NaCl (saline). This isolation procedure was repeated twice to obtain a purified bacterial target.

3.2.3. Preparation of bacteria-doped PEDOT films

A sheet of indium tin oxide (ITO)-coated glass (resistance: 10 Ω cm⁻²) was cut into a 0.6 × 1.9-cm strip and sonicated in ethanol for 30 min. This ITO glass strip, an Ag|AgCl|saturated KCl|| electrode, and a platinum sheet (0.7 × 1.5 cm²) were employed as the working, reference, and counter electrodes, respectively. The electrode potential was referred to the above reference electrode throughout this report. The electrodes were placed in a glass cell, which contained 10 mL of aqueous solution consisting of 10 mM EDOT, purified bacteria (2.6×10⁷ cells mL⁻¹), 90 mM phosphate buffer (pH 7), and 0.020 mM NaCl. A constant potential (1.0–1.2 V) was applied for a given time (450–1200 s) to deposit a PEDOT film on the ITO electrode. Freshly polymerized PEDOT/bacteria films were then rinsed with an ample amount of water and immediately subjected to voltammetric and microscopic analyses.
3.2.4. Microscopic observation

The PEDOT/bacteria film was stained with fluorescence pigments, SYTO9 and propidium iodide (PI), according to the manufacturer’s instructions for the Bacterial Viability Kit (Molecular Probes). Bacteria immobilized in the PEDOT films were observed under a fluorescent microscope (BX51, Olympus Co.; Japan). The surfaces of the polymer films were also evaluated by scanning electron microscopy (SEM; TM-1000, Hitachi; Japan). To estimate the film thickness by observing cross-sectional SEM images, the ITO glass coated with the PEDOT/bacteria film was set at an angle of 90° against the sample holder.

3.2.5. Electrochemical analysis

Cyclic voltammetry (CV) of the PEDOT films was performed in a scanning range from −0.7 to +0.5 V in 90 mM phosphate buffer solution (pH 7.0) at a scan rate of 10 mV s\(^{-1}\) using an automatic polarization system (µAutolab-TypeIII, Metrohm Autolab; the Netherlands). All the voltammetric experiments were conducted under a nitrogen atmosphere otherwise noted.

3.3. Results and Discussion

3.3.1. Electrochemical characterization

The applied potential and polymerization time have primary importance in the electrochemical features of the resulting films; thus, the PEDOT/bacteria films were carefully prepared by varying these parameters to evaluate their effects.

To maintain neutral pH, which favors the living condition of bacteria, the polymer film was originally prepared in an aqueous monomer solution, the pH of which was adjusted using phosphate buffer at pH 7 in the presence and absence of bacteria (Figure 3-1 a). Under this condition, the polymer film was competitively doped with both the bacteria and anionic components in the phosphate buffer (HPO\(_4^{2-}\) and H\(_2\)PO\(_4^-\)). Hereafter, the films were designated as PEDOT/phosphate and PEDOT/bacteria, although the latter film was also doped with the phosphates. The pair of redox peaks at approximately −0.35 V and −0.08 V for both the PEDOT/phosphate and PEDOT/\(P.\ aeruginosa\) films indicated that they could be assigned to the redox event of the polymer, i.e., doping and dedoping of phosphates. The much smaller size of phosphates than that of bacteria allows these anions to be the main species traversing the film interface during the polymer redox event.
The above observation led to the finding of a condition that would minimize these polymer redox peaks for effective monitoring of the bacterial activity. Since it has been reported that phosphate as a dopant results in clear redox peaks, it was obviously not the appropriate electrolyte for targeting purpose. It has been found that a very small concentration of NaCl (0.02 mM) added during polymerization instead of phosphate satisfied the condition. The voltammograms of the PEDOT films doped with Cl\(^-\) and \textit{P. aeruginosa}\ are shown in Figure 3-1 b, in which the peaks assigned to the polymer redox were mostly removed. The pair of reduction and oxidation peaks appeared at \(-0.5\) V and \(-0.4\) V, respectively, and was therefore attributed to a redox event related to the bacteria. According to the literature, the redox processes of bacteria are derived either from redox mediators, which are secreted in the metabolic process of bacteria, or from redox enzymes located on the bacterial cell’s envelope, or from both.\(^6,7\) \textit{E. coli}, another negative charge-carrying bacterium, was also immobilized into the PEDOT film, and performed an electrochemical response very similar to that of the PEDOT/\textit{P. aeruginosa} film (Figure 3-1c). The higher current density for the \textit{E. coli} film could be explained if such mediators and/or enzymes were more concentrated in the film than those for the \textit{P. aeruginosa} film.

\begin{figure*}[h]
\centering
\includegraphics[width=\textwidth]{figure31.png}
\caption{Cyclic voltammetry of the poly(3,4-ethylenedioxythiophene) (PEDOT) films doped with (a, b) \textit{P. aeruginosa} (PEDOT/P.a) and (c) \textit{E. coli} (PEDOT/E. coli) recorded in phosphate buffer at pH 7 and at a scan rate of 10 mV s\(^-1\). PEDOT films were electrochemically deposited on ITO electrodes at 1.05 V for 900 s in aqueous solution (10 mL), which consisted of 10 mM EDOT, 2.6\times10\(^7\) cells mL\(^{-1}\) bacteria, and an electrolyte, i.e., (a) 90 mM phosphate buffer (pH 7); (b and c) 0.020 mM NaCl, respectively. The bacteria were not added in the preparation of the control films (break line), PEDOTs doped with chloride and phosphate.}
\end{figure*}
The observed difference in the voltammograms between bacteria adsorbed on the PEDOT film and doped in the film indicated the importance of their roles in the redox event. Prior to the CV measurement, a *P. aeruginosa* suspension (0.1 mL, 2.6×10⁷ cells mL⁻¹) was dropped on the PEDOT/Cl⁻ film (about 1 cm²), which was then left for 30 min to allow the attachment of the bacteria on the polymer film. Little change in the electrochemical response of the PEDOT film with adsorbed bacteria (data not shown) suggested that the bacteria simply adsorbed on the film did not activate the redox event.

The stability of PEDOT/*P. aeruginosa* film was demonstrated by the fact that this polymer could be electrochemically cycled without losing its redox activity. The current slightly decreased until the steady state was reached after 30 cycles (Figure 3-2).

Figure 3-3 demonstrates the dependence of the current density at −0.15 V and thickness of the PEDOT/*P. aeruginosa* film on the polymerization potential and polymerization time. The current density linearly increased with the potential (Fig. 3-3 a) as well as with polymerization time (Fig. 3-3 b). Since a linear thickening of the PEDOT film with respect to the polymerization parameters was expected from these results, the thickness of the films that were grown by changing either of these parameters was measured using SEM (Fig. 3-3 c&d). The thickness of the PEDOT/*E. coli* films prepared in this study ranged from 0.3 to 1.5 µm.

![Graph](image)

**Figure 3-2.** CVs of the PEDOT/*P. aeruginosa* film in a pH 7.0 phosphate buffer at scan rate of 100 mV s⁻¹. PEDOT was electrochemically deposited on an ITO electrode at 1.05 V for 900s in an aqueous solution (10 mL), which consisted of 10 mM EDOT, bacteria (2.6×10⁷ CFU mL⁻¹) and 0.020 mM NaCl.
Figure 3-3. Charging current and thickness of the poly(3,4-ethylenedioxythiophene) (PEDOT) / \textit{P. aeruginosa} films under different polymerization conditions: (a) the current density of the films polymerized for 450 s at the given potentials, (b) the current density of the films polymerized at 1.05 V for the given times, (c) thickness of the PEDOT films prepared in the experimental series of a, and (d) thickness of the PEDOT films prepared in the experimental series of b. The thickness values were obtained from SEM observations.

3.3.2. Viability of bacteria doped in the polymer film

Figure 3-4 shows the SEM images of the PEDOT/\textit{P. aeruginosa} film and demonstrates the state of the bacteria located at the film surface. The contrast changes arising from the difference in the conductivity of the film components indicate that the film base, which appears in gray color, was a conductor, whereas the black-colored bacteria were insulators. Most of the \textit{P. aeruginosa} showed a rod-like shape with an average dimension of 0.6 µm in width and 3 µm in length, and their density was estimated as $1.1 \times 10^4$ cells cm$^{-2}$. These results, as well as the electrochemical characteristics of the PEDOT films, confirmed that the bacteria acted as dopants during the electrochemical polymerization.
Figure 3-4. Scanning electron microscopy images of the poly(3,4-ethylenedioxythiophene) (PEDOT)/P. aeruginosa film polymerized at 1.05 V for 900 s. The electrode was fixed on the sample holder at an angle of 60°. Inset: enlarged view.

To determine whether the bacteria were viable during and after doping, fluorescence microscopy experiments were conducted. The living and dead bacteria were assayed using the SYTO9/PI fluorochromes, which differentially stained the bacteria; SYTO9 labels all the bacteria in a population, including both dead and living cells, to release a green signal, whereas PI only stains dead cells with red color. These fluorochromes were added to the bacteria after the polymerization procedure. To evaluate the effect of the PEDOT layer on cell viability, the bacterial suspension was applied onto a bare ITO electrode to allow direct contact between the bacteria and the oxide surface; the electrode was then polarized at 1.05 V for 900 s and subsequently stained. Figure 3-5 a shows the fluorescent images of the bacteria adsorbed onto the bare ITO electrode. Red cells (right side) represented almost the entire population (left side), indicating that most bacteria were dead after the contact (approximately 0% viability). This was probably due to the electrochemical or adsorbing process impairing the survival of these cells. Approximately 20% of P. aeruginosa cells remained viable on the thin PEDOT film, which showed a thickness of approximately 0.4 µm (Figure 3-5 b), while more than 90% of the cells were viable on thicker films (>0.6 µm; Figure 3-5 c). Although the polymerization potential and time did not affect the bacterial viability on the PEDOT film, the direct contact of P. aeruginosa cells with the ITO glass, which had been vapor-coated with metal and metal oxides, significantly impaired their survival rate. Compared to P. aeruginosa, E. coli appeared to be a more robust bacterium, with approximately 60% of the cells remaining alive in the thin (0.3 µm) PEDOT film (data not shown). However, this rate also increased with the thickening of the polymer film, reaching almost 100%.
Figure 3-5. Fluorescence microscopy images of bacteria stained with SYTO9 (left) and by propidium iodide (PI) (right). In panel a, bacteria were directly applied onto a bare ITO electrode and then polarized at 1.05 V for 900 s. In panels b and c, bacteria were fixed on/in the poly(3,4-ethylenedioxythiophene) (PEDOT) films, which were prepared with the monomer at 1.05 V for (b) 450 s and (c) 900 s.

In contrast to polyaniline, which could only be synthesized in an acidic medium, PEDOT was efficiently polymerized even at neutral pH, which provided more favorable conditions for the bacteria during inclusion. Therefore, this one-step immobilization technique ensures very high viability; moreover, PEDOT plays a significant role in maintaining high bacterial viability, providing excellent biocompatibility.

Figure 3-6. Relationship between bacterial viability and film thickness. The films were prepared (○) for 450 s by varying the polymerization potential, and (▲) at 1.05 V by varying the polymerization time.

Figure 3-6 illustrates the relationship between the viability of entrapped P. aeruginosa and film thickness. The film thickness was controlled either by the polymerization potential or by the polymerization time. In the conditions corresponding to the film thickness of <0.5 µm, only
20% of bacteria were viable. However, a dramatic increase in viability was observed in films thicker than 0.5 µm, with a final viability of >90%. These results confirmed that the film thickness is an important factor in controlling the bacterial viability, which is critically affected by a thickness value of 0.5 µm.

At the initial stage of the polymerization, most bacteria died due to their direct interaction with the electrode (thickness < 0.5 µm). Continuing polymerization produced a film with low viability (thickness ~0.5 µm), which included both dead and live bacteria i.e., the viability observed during this period depended on the thickness of the PEDOT layer deposited underneath each bacterium (Fig. 3-5 b). Further polymerization resulted in a thickness of more than 0.5 µm to generate a floor suitable for bacterial survival. It should be noted that the green cells at the film surface were all alive, since the cells embedded under the surface were not stained due to the small diffusion coefficients of the dyes toward the film (Fig. 3-5 c); thus, the viability was measured as >90%. The film structure was supported by the SEM observation, which demonstrated the doping state of the bacteria inside and on the PEDOT film (Fig. 3-4).

The results shown in Figure 3-5 demonstrate that the PEDOT/P. aeruginosa film grew and doped bacteria in two different stages. Previously reported techniques for the immobilization of bacteria through physical adsorption required covering the surface with a permeable membrane to prevent cell leakage.8–10 In contrast, technique used in this report allows high bacterial activity without requiring such a modification step. This technique also provides a high density of bacteria with strong interactions between the negatively charged bacterial outer membrane and the polymer backbone.

The electrochemical stability of bacteria after the immobilization was evaluated based on the viability after potential cycling between -0.7 V to +0.5 V in the pH 7 phosphate buffer. In comparison to P.aeruginosa and S.oneidensis, E.coli was more robust, and its viability was almost constant during the voltammetric experiment (Figure 3-7). However, after 30 scanning cycles, more than 80% of the doped P. aeruginosa were found dead. To make the reason clear for the high fatality rate, scanning was divided to the anodic and cathodic directions. As a result, the 30-scanning cycles between 0.0 V and -0.7 V killed about 80% of the P. aeruginosa, while the cycles between 0.0 V and +0.5 V killed only 10% of bacteria. Accordingly, it was turned out that only the cathodic scan damaged the bacteria. Hydrogen peroxide generated by the reduction of dissolved oxygen cannot be the reason for the low viability, since there was no difference in the viability between the experiments carried out under the oxygen and nitrogen atmosphere.
(compare the closed symbols of *P. aeruginosa* to the round ones). A likely reason for the low viability is that the bacterial cell membrane is damaged by the scan. It is supposed that the voltage stimulus impairs certain components of the cell membrane, such as lipid bilayers and the membrane bound proteins.

![Graph](image)

**Figure 3-7.** The dependence of the viability of (●, ○) *P. aeruginosa*, (▲) *E. coli* and (◆) *S. oneidensis*, doped in the PEDOT films, on the scan cycle at pH 7. Voltammetry was conducted under aerobic conditions for (●, ▲, ◆), while it was done under the anaerobic condition for (○). The polymer films were scanned in the range of -0.7 V to 0.5 V at 100 mV s⁻¹.

### 3.3.3 Application of the bacteria-doped films

*P. aeruginosa* is a methane-oxidizing bacterium, which is able to use methane as the carbon source along with oxygen consumption. Recently, these bacteria have been utilized to fabricate a methane-biosensing system by immobilization in a polyvinyl alcohol-alginate complex.¹¹ The technique employed in the current study is promising for this kind of application because of the simplicity of the single-step preparation and excellent biocompatibility.

In addition to *P. aeruginosa* and *E. coli*, *S. oneidensis*, a redox bacterium, was also immobilized into the PEDOT film during electrochemical polymerization at 1.05 V for 900 s. *S. oneidensis* was easily doped into the PEDOT film owing to the negatively charged groups located on its outer membrane, and maintained high viability (90%). The PEDOT/*S. oneidensis* film showed a similar chemical response with two identical redox peaks to those of the PEDOT/*P. aeruginosa* and PEDOT/*E. coli* films (Fig. 3-8). The close separation of the reduction and oxidation peaks at −0.5 V and −0.4 V, respectively, resulted from the fast electron transfer of the redox compounds originating from the bacteria doped on the polymer film. This behavior suggests that these PEDOT/bacteria films are potential candidates for use in microbial fuel cells, in which fast electron transfer is required to yield high current output.
Figure 3-8. Cyclic voltammetry of the poly(3,4-ethylenedioxythiophene) (PEDOT) film and PEDOT film doped with *S. oneidensis* (PEDOT/S.o) in phosphate buffer (pH 7) at a scan rate of 10 mV s⁻¹. PEDOT was electrochemically deposited on an ITO electrode at 1.05 V for 900 s in an aqueous solution (10 mL), which consisted of 10 mM EDOT, 0.020 mM NaCl in the presence or absence of bacteria (2.6×10⁷ cells mL⁻¹).

The bacteria evaluated in the present study showed redox activity under anaerobic conditions. In contrast to *S. oneidensis*, whose outer membrane-bound cytochromes and excreted mediators are involved in electron transfer (Fig. 3-8), this process in *P. aeruginosa* and *E. coli* is widely reported to be due to the excreted mediators. Moreover, low levels of cytochromes and redox proteins in the outer membrane compared to those in the cytoplasmic (inner) membrane of *P. aeruginosa* and *E. coli* were also reported. Therefore, in the current study, it is likely that only the redox mediators excreted by the bacteria generated the reduction and oxidation peaks (Fig. 3-1). Flavin (*E⁰ = −0.43 V*), phenazines (*E⁰ = −0.55 V*), or quinones (*E⁰ = −0.41 V*) appeared to be suitable candidates due to their close redox potential values to the redox peaks in this study (*E_m = −0.45 V*). Ubiquinones carrying isoprenoid-chains, which exist in bacterial cells have been reported to show sharp cathodic and broad anodic peaks when inserted in a lipid bilayer structured on a gold electrode. Similar cyclic voltammetric responses of the PEDOT/bacteria film suggest that the ubiquinones are the most likely candidate. However, further investigation is necessary to reach the conclusion.

3.4. Conclusion

PEDOT/bacteria films were successfully prepared in a thin and transparent state, being a favorable substrate for entrapping bacteria as maintaining the viability up to 98%. It is noteworthy that this high viability was reached at a film thickness above 0.5 µm. Investigation on the viability of doped bacteria against the voltage stimulus revealed that *P. aeruginosa* and *S. oneidensis* were stable for anodic scans (from 0.0 V to +0.5 V), while the cathodic scans...
(between 0.0 V and -0.7 V) killed about 80% of bacteria after 30 scan cycles. However, no such deterioration was observed for the voltammetric response of PEDOT/E. coli. The PEDOT/bacteria film performed an excellent stability as not losing redox activity after 30 cycles of electrochemical scanning.
REFRENCES


CHAPTER IV

Development of a Detecting Technique based on Voltammetric Responses of Quinones Hydrophobically Transferred from Deposited Bacterial Cell

4.1. Introduction

The detection of microorganisms plays a crucial role in many bio-related fields, such as the food, medical and environmental industries. An accurate and cost-effective detection technique making real-time quantification possible is still an important goal to be achieved. Culture and colony counting have been established as a reliable and accurate techniques. However, the overall procedure is time consuming, extending sometimes to a week, and labor-intensive. A number of techniques in bacterial detection aimed at replacing the culture technique have been presented, and, concerning electroanalysis, several types of biosensors, such as immunosensors, genosensors and aptasensors have been reported due to their selectivity and sensitivity against targeted bacteria as well as the abundance in available detection formats.\textsuperscript{1-7} In this connection, a quartz-microbalance (QCM) based real-time bacterial sensor using a polymer film imprinted with the targeted bacteria has been developed.\textsuperscript{8,9} However, as far as field applications are concerned, QCM detectors have some issues to be resolved regarding externally induced mechanical vibrations and a high per-chip cost as well as a large overall instrument footprint in mobile applications. The initial motivation of this work is therefore to develop a cost-effective and robust bacterial detecting device based on an electrochemical technique in order to circumvent these problems.

Accordingly, direct voltammetry using electrodes with immobilized microorganisms is objective of this study. Matsunaga et al. reported that Gram-positive bacteria (\textit{Lactobacillus acidophilus}) and Gram-negative (\textit{Escherichia coli}) bacteria transferred onto basal plane pyrolytic graphite and gold electrodes produced an anodic peak capable of detecting the cells down to $10^{8}$ cells cm$^{-2}$.\textsuperscript{10-12} They suggested that the mediation with coenzyme A present in the cell wall was responsible for the current responses. However, a large background current observed in this direct method would lead to a difficulty in an application requiring a highly sensitive detection. A direct correlation was made between the cell concentration and the redox current of dye molecules interacting with bacterial cells. Studies demonstrated that Safranin O, 5-cyano-2, 3-ditolyl tetrazolium chloride, and propidium iodide (PI) could be used as
electrochemical probes for the detection of E. coli and P. aeruginosa. Mycolic acids, found in the cell envelopes of Mycobacterium, Gordona, Nocardia and Rhodococcus, were extracted by organic solvents, and subjected to voltammetric analysis to provide a detection limit of $5.9 \times 10^2$ CFU mL$^{-1}$.14

Apart from electroanalysis, bacteria-immobilized electrodes have been extensively studied in the field of microbial fuel cells (MFCs), which culture microorganisms on the anodes to generate the electric energy as a result of their respiratory action (Scheme 4-1 A).15-17 Many species of anaerobic bacteria either directly or indirectly eject electrons to terminal electron accepters, such as anodes,17-20 but usually give ill-defined voltammograms with a few exceptions, such as the Geobacter sulfurreducens strains.19-21 In addition, the extended time requiring days until the full growth of biofilms on the anodes makes their real-time applications difficult.

In this chapter, a report of a rapid detection technique of bacteria by damaging bacterial cell walls by desiccation, which is a reverse way of culturing used in the MFC studies, will be given. By adopting this technique, the signals generated from isoprenoid quinones in microorganisms can be detected without using any chemicals to extract these marker molecules. In addition, the electrode gives information on the levels of the isoprenoid quinones, which play important roles in electron and proton transfer in microorganisms.

4.2. Experimental

4.2.1. Materials

All chemicals were of pure analytical grade and were used as received. Millipore quality water was autoclaved before use. Genetically modified verotoxin-nonproducing E. coli PV856 (O157:H7) and E. coli PV276 (O157:HNM) were provided by Prof. M. Miyake, Osaka Prefecture University and Dr. K. Seto, Osaka Prefectural Institute of Public Health. E. coli K-12 (O Rough:H48, NBRC3301), Pseudomonas aeruginosa (NBRC3080), Vibrio parahaemolyticus (NBRC12711), Salmonella enterica (NBRC13245), Staphylococcus aureus (NBRC102135), Rhodobacter sphaeroides (NBRC12203), and Corynebacterium glutamicum (NBRC12169) were purchased from the Biological Resource Center, Japan, while Shewanella oneidensis MR-1 (ATCC700550) was acquired from the American Type Culture Collection, U.S.A. In this study, the K-12 strain is denoted as E. coli unless otherwise noted. Bacillus
subtilis var. natto and coliform bacteria were isolated from natto (fermented soybeans) and prawns, which were purchased at local supermarkets, respectively.

4.2.2. Cultivation, purification, and characterization of bacteria

Cultivation. The inocula used for the cultivations were obtained from single colonies on agar plates. All the culturing media were autoclaved before use. Cultures were aerobic and batchwise in an airtight plastic tube at 303 K for 18 h, unless otherwise noted: All the bacteria evaluated in this study except V. parahaemolyticus were cultured in Nutrient Broth (abbreviated NB; EIKEN CHEMICAL Co. Ltd., Japan), while V. parahaemolyticus was cultured in Marine Broth 2216 (Difco Laboratories, Ltd., Japan).

Isolation and culture of B. subtilis var. natto and coliform bacteria were as follows: A few pieces of natto beans were placed in 15 mL of boiling water, and this mixture was allowed to stand until returning to room temperature. An inoculating loop, which had been dipped in the mixture, was used to streak the bacteria on agar plates of Luria-Bertani media. The plates were incubated overnight at 303 K for picking up single colonies, which were then cultured overnight in NB at 303 K. Coliform bacteria were cultured from law prawn meat following the procedures documented in the regulatory standards of the Ministry of Health Labour and Welfare, Japan (Bulletin No. 370, 1979).

Aerobic and anaerobic cultures for analysis of dynamical profiling of quinones. The aerobic culture was conducted as follows: 15-mL of NB containing 0.25% glucose was transferred to a plastic tube (50 mL), then autoclaved. The bacteria were then inoculated in the tube. The tube was incubated under shaking culture conditions at 303 K for 18 h. The anaerobic culture was done as follows: The bacteria suspension of 150 mL was prepared with the same composition as used in the aerobic culture, and transferred to a 150 mL plastic tube. The plastic cap was tightly closed during the static cultivation (303 K, 18 h).

Purification. All the cultured bacteria were purified as follows: A 15-mL portion of the cultured suspension was centrifuged at 6,000 rpm for 7 min at 277 K, and the precipitate was resuspended in 15 mL of saline (0.85% NaCl). The procedures were repeated twice to obtain a purified cell pellet. The pellet resuspended with 2–3 mL of water gave OD_{600} values of 20–30. Either a 5- or 10-µL aliquot of the suspension was used for the electrode preparation, while the rest was used to determine the bacterial population by its OD_{600} value; namely, the OD value was correlated to the cell count with a 3M Petrifilm (3M Health Care, Ltd.) to evaluate the
bacterial density on an ITO electrode. Typical values were: 1 OD$_{600}$ $= 6.2 \times 10^8$ and $1.4 \times 10^8$ cells mL$^{-1}$ for *E. coli* and *S. oneidensis*, respectively.

**Characterization of coliform bacteria isolated from prawn meet.** Single colonies were re-isolated from the bacterial smear grown on the EMB agar plates. Eight to twelve colonies/sample grown on EMB or LB agar plates at 310 K for 16-24 h were isolated (32 colonies in total) for further investigation. Taxonomic examination was performed by determination of the 16S ribosomal RNA sequences. Small aliquots of the bacterial pellet from each colony were suspended in sterilized distilled water, and lysed by the heat treatment at 368 K for 5 min. After centrifugation at 10,000 $\times$ g for 5 min, the supernatants were used as templates for the polymerase chain reaction (PCR) using a primer pair (5’-CAg ACT CCT ACg ggA ggC AgC AgT and 5’-CgT ggA CTA CCA ggg TAT CTA ATC CTg TTT gC) amplifying gene fragments containing region V3 to region V4 of 16S ribosomal RNA. The DNA sequences of the PCR products were determined by the Sanger dideoxy sequencing method from Macrogen Japan Co. (Japan). The obtained sequence reads were first analyzed by ClustalW to examine the phylogenetic relationships, and then by a BLAST search to examine the homology with published bacterial 16S ribosomal RNA sequences. These experiments were kindly conducted by Prof. Masami Miyake (Department of Veterinary Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University).

**4.2.3. Quinone extraction**

The solvent extraction of quinones were conducted by the following procedures: A 2.0 mL volume of a 2:1 chloroform/methanol mixture was added to the purified *E. coli* pellet obtained from 15 mL of the cultured NB. The mixture was sonicated for 15 min and then centrifuged at 6,200 rpm for 10 min to collect the supernatant (extract A). Another chloroform/methanol solution of 2.0 mL was added to the precipitate, and this mixture was vigorously shaken and centrifuged at 6,200 rpm for 10 min. The supernatant was then combined with extract A. The entire supernatant was filtered using a membrane filter (0.2 µm) and stored at 253K.

**4.2.4. Procedures and instrumentation**

All microbial experiments were performed under strictly sterile conditions. A Ag|AgCl|saturated KCl electrode and a platinum mesh electrode (1 cm$^2$) served as the reference and counter electrodes, respectively, throughout this study. All the potential values reported in this study are referred to this reference electrode, unless otherwise noted. An indium-tin-oxide
(ITO) glass strip with the dimensions of 26 mm × 77 mm × 1 mm, obtained from Kinoene Optics Co., Japan, was used as the working electrode (10 Ω cm⁻²). The surface of this strip was covered with a UV curing resin film (EUV-CY ink, Roland DG, Japan) using a Roland DG LEF12 inkjet printer except for a circular area used as the working electrode (2.0 mm or 4.0 mm in diameter). Before use, the electrode was either sonicated in 50% ethanol-water for 5 min, or washed with water; both the procedures gave the identical results. A glassy carbon electrode (1.6 mm diameter, BAS Co., Japan) polished with alumina powder was also used for comparison purposes.

Voltammetric experiments were performed as follows: The purified bacteria dispersed in 5.0- or 10-µL of water were dispensed on the surface of the 2.0-mm or 4.0-mm diameter ITO electrode, respectively; the 4-mm electrode was mostly used in this study. In the case of the glassy carbon electrode, a bacterial suspension of 2 µL was applied. After the application of the bacterial suspension, the electrode was put into a desiccator maintained at room temperature (294–300 K) and at a relative humidity of ~24 %, until the surface was completely dry (30–60 min, depending on the suspension volume and room temperature). Voltammograms were recorded in a pH-7.0 phosphate buffer (ionic strength, 0.2) at 20 mV s⁻¹ under a nitrogen atmosphere, unless otherwise noted. The measurements were made in a Faraday cage (BAS Model TB-1) thermostated at 303 K with an ALS CHi842B Electrochemical Analyzer.

Detection efficiency of the isoprenoid quinone obtained by the present technique was compared to that for the solvent extraction. Differential equations formulated for adsorption voltammetry were numerically solved using Wolfram Mathematica 10 software. Bacterial cells were stained and counted to evaluate the viability and density on the electrode using SYTO 9/PI fluorochrome reagents (LIVE/DEAD BacLight Bacterial viability Kit L7007, Thermo Fisher Scientific, Inc.) after the electrode desiccation. The ITO electrode surfaces were imaged using a fluorescent microscope (ECLIPSE Ni, Nikon, Japan) and a scanning electron microscope (SEM; Miniscope TM3030, Hitachi, Japan).

4.3. Results and Discussion

4.3.1. Voltammetric responses after in-situ evaporation of E. coli suspension

Figure 4-1 shows the development of evaporation on an ITO electrode, on which the E. coli suspension was applied. An ITO glass strip coated with the polymer resin was used as the working electrode (A). A bacterial suspension of 10 µL was applied (C) on the 4-mm
diameter electrodes and dried for 60 min (E). The electrode prepared in this way was subjected to voltammetric analysis in phosphate buffer (pH 7.0 in most cases).

Figure 4-1. Preparation of a bacteria-adsorbed ITO electrode (diameter, 4 mm) by dispensing 10 µL of an *E. coli* suspension (2.0×10^{10} cells mL^{-1}): Top views of the electrode; (A) before and (B) after application of the bacteria suspension. In (B) the dried area is bright, while the wet area is dark. Time elapsed after dispensing the suspension at 296 K was (B) 58 min, (C) 0 min, (D) 45 min, (E) 60 min. The electrode was not placed in the desiccator while taking these photographs, so that the time line in this figure does not exactly match with that in Fig.4-2 due to differences in temperature and humidity.

Figure 4-2. Voltammetric responses of an ITO electrode (diameter, 4 mm) depositing *E. coli* (1.5×10^{9} cells cm^{-2}) for the different evaporation times at 60 mV s^{-1} in a pH 7.0 phosphate buffer under anaerobic conditions at 298 K. Temperature and relative humidity during the desiccation were 295 K and 24 %, respectively: (A) Cyclic voltammograms after a desiccation time of (a) 32 min and (b) 45 min. (B) The current density of (●) the first cathodic peak, c1, and (○) the second cathodic peak, c2, as a function of the desiccation time.
As long as the entire surface remained wet (≤32 min), no redox peaks were observed. When a dry area emerged on the rim of the electrode (Fig. 4-1B), two pairs of redox peaks appeared in the voltammogram, followed by continuous growth until the surface reached complete exsiccation; this time profile is shown in Figure 4-2. There seems to be two reasons for the emergence, which is discussed in more detail in the following section: (1) bacteria might be forced to excrete a biofilm, in which mediators for extracellular electron transfer are involved, to maintain moisture for their survival, and (2) the fractional breakdown of bacterial cells occurring during dehydration leads to a release of redox active proteins and/or mediators onto the electrode (Scheme 4-1).

Figure 4-3. CVs of the E. coli-deposited electrodes: (A) the ITO culturing E. coli (2.7 × 10^6 cells cm^-2), (B) the E. coli (2.3 × 10^9 cells cm^-2)-deposited GC, (C) the E. coli (2.1 × 10^9 cells cm^-2)-deposited ITO, and (D) the ITO applying 3.0 µL of the bacteria-removed NB broth. Curves (1) are for the bare electrodes, and (2) are for the bacteria-deposited electrodes. All the CVs were recorded in a pH-7.0 phosphate buffer at scan rates of 20 mV s^-1 for (A-C), and 60 mV s^-1 for (D).

Figure 4-3 shows four different systems to characterize the E. coli-based voltammetric responses. Voltammogram (A) shows the response of E. coli grown on an ITO electrode. First, the electrode was placed at the bottom of a cylindrical glass cell and the bacteria suspended in Nutrient Broth (NB) were added to the cell to start a static culture at 303 K. After three days, the electrode was rinsed with water and voltammetrically examined in the pH-7.0 phosphate buffer. Although SEM observation showed that the cells on the ITO surface were relatively sparse (~3×10^6 cells cm^-2), the difficulty in removing the cells by a rinse of the electrode with water indicated that the cells were fixed on the surface by forming a biofilm. This preparation
technique is therefore similar to that used during preconditioning in MFCs, but the result was just a pair of ill-defined peaks.

Voltammogram (B) was recorded using a glassy carbon electrode prepared by evaporation; a 2.0-µL volume of a *E. coli* suspension was dispensed on the electrode (1.6 mm diameter) and dried in the same way as discussed in Fig. 4-1. However, voltammetric examination in the phosphate buffer only produced two pairs of partially resolved redox peaks. In contrast, an ITO electrode (C) revealed a much better resolution, though prepared in the same way as (B). The electrode (D), which was prepared by evaporating the NB medium that had been used in culture, did not show any appreciable peaks; in this experiment, the culture was centrifuged to precipitate bacteria and its supernatant was subsequently applied on the electrode. The absence of the peak in (D) denied the possibility that the peaks in (C) resulted from either chemical components involved in the broth or metabolites during the culture. Accordingly, it can be safely concluded that the bacterial deposition was responsible for the appearance of the voltammetric peaks.

**Figure 4-4.** SEM images of the ITO glass surfaces depositing *E. coli* of 4.9 × 10^8 cells cm^2^, (A) after desiccating for 40 min, (B) after immersion for 15 min in the phosphate buffer, (C) after CV at 60 mV s^−1^, and (D) after heat desiccation.

Figure 4-4 shows SEM images of the ITO electrode surfaces after depositing the *E. coli*. Image (A) shows the electrode immediately after the evaporation of the suspension in the desiccator, and the dense black areas indicate stacked bacterial layers. This electrode was submerged in the phosphate buffer for 15 min, during which N\textsubscript{2} gas was bubbled through, to reveal that this stacked structure was removed (B). After the voltammetric measurement (C),
the cells were found with a lower contrast, suggesting that their cytosols would have been lost presumably due to the damage in the cell membrane caused by the voltage stimulus. When the electrode was dried by heat, the bacteria clearly discharged their internal fluids, with which they were encompassed (D). The electrode after the voltammetric cycle gave images similar to those for (C).

Viabilities obtained by staining the cells were $37 \pm 7\%$, $30 \pm 2\%$, $6 \pm 4\%$, and $0\%$ for (A), (B), (C) and (D), respectively. Thus, about $30\%$ of the cells had survived after the desiccation, but most of them were damaged by the voltage stimulus during the first voltammetric cycle (Scheme 4-1), which agreed with the SEM image of the dysfunctional cells shown in (C).

**Scheme 4-1.** Model illustrations of a Gram-negative bacterial cell on an electrode

![Scheme 4-1](image)

(A) a cross sectional view of an intact cell on the electrode,\textsuperscript{12,13} (B) a breakdown of the outer membrane due to dehydration, and (C) hydrophobic transfer of isoprenoid quinones after potential cycling. The ubiquinol/ubiquinone and menaquinol/menaquinone couples are denoted as UQH\textsubscript{2}/UQ, and MKH\textsubscript{2}/MK, respectively.
4.3.2. Voltammetric behavior of E. coli deposited on ITO electrodes

ITO electrodes depositing E. coli were examined at different initial and switching potentials. The linear dependence of all the four peaks on the scan rate suggested that the peaks arose from the surface-confined redox reactions (Fig. 4-5). Panel (A) in Figure 4-6 indicates that the peaks, namely, c1/a1 and c2/a2, are assigned to two different reversible redox couples. The invariant height of each peak after repeated scan cycles indicated that these compounds were stable against the redox reactions. The second cycle in voltammogram (B) also shows that the c2/a2 couple is electrochemically independent of the c1/a1 couple. Based on these observations, it is clear that the E. coli left two different reversible compounds on the electrode surface.

Figure 4-5. Dependence of the peak currents on the scan rate for S. oneidensis (2.3×10^8 cells cm^{-2}) at an ITO electrode (diameter: 4 mm) at 303 K.

Figure 4-6. Voltammetric responses of the E. coli (1.4×10^9 cells cm^{-2})-deposited ITO electrode recorded in different potential range (A) between -0.4 and 0.6 V, and (B) between -0.7 and 0.2 V in a pH-7.0 phosphate buffer at 20 mV s^{-1}. 
4.3.3. Assignment of the voltammetric peaks

Several candidates have been proposed as the electron mediators that the bacteria used in MFCs secrete.\textsuperscript{22-24} Mechanistic studies of the electron transfer reactions in MFCs indicate that electrons are either directly or indirectly transferred from bacteria to anodes via membrane proteins, i.e., outer membrane-associated c-type decaheme cytochromes, OmcA, and MtrC (e.g. Scheme 1A).\textsuperscript{17}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure4_7.png}
\caption{Voltammetry of ITO electrodes depositing \textit{E. coli} and \textit{S. oneidensis}. (A) The voltammogram for \textit{E. coli} was recorded using electrode not treated with GuHCl but adjusted to pH 3.5 with phosphate. The voltammograms of (B) \textit{E. Coli} and (C) \textit{S. oneidensis} were recorded using electrodes treated with 7 M guanidine monohydrochloride (GuHCl) for 15 min (pH 3.5) before observation in this medium. The curves, 1 and 2, in (B) were for the responses with and without \textit{E. coli}, respectively. Scan rate, 20 mV s\textsuperscript{-1}; bacterial density, 2.1×10\textsuperscript{9} cells cm\textsuperscript{-2} for \textit{E. coli}, and 2.8×10\textsuperscript{8} cells cm\textsuperscript{-2} for \textit{S. oneidensis}.}
\end{figure}

To determine if these proteins are involved in the voltammetric response, denaturing experiments were done using guanidine. It has been reported that the addition of guanidine leads to the disappearance of the voltammogram of cytochrome c due to denaturation.\textsuperscript{25} The results of the guanidine addition experiments suggest that these proteins were not involved in the CV of the bacterial deposits (Fig. 4-7). The electrodes having \textit{E. coli} and \textit{S. oneidensis} were treated with guanidine to see if the surface proteins were involved in the voltammetric responses. The guanidine hydrochloride solution was pH 3.5 at 7 M. Although it is not very clear from the observation of the cathodic branch due to the emergence of an overlapping broad peak, the
presence of two anodic peaks found unaffected by guanidine suggests that the proteins were not involved in the redox events at the evaporated electrodes.

To explore other possibilities that are responsible for the voltammetric peaks, the voltammetric responses of flavin adenine dinucleotide (FAD), pyocyanin, ubiquinone (UQn), and menaquinone (MKn), which have been reported in MFC studies as secretions from bacteria were studied.\textsuperscript{22-24} It was firstly examined the voltammetric responses of soluble electron mediators, FAD and pyocyanin. As expected, these compounds only exhibited diffusional redox responses without giving any sign of the symmetric adsorption peaks, and it was confirmed that they were not the origin of the redox peaks.

The \textit{E. coli}-deposited ITO electrode that had been repeatedly washed with water produced the voltammetric peaks to the same extent as before washing. However, the electrode that had been rinsed with a 2:1 chloroform/methanol mixture did not show any detectable peaks due to the removal of the adsorbed compounds. This result indicated that two compounds were hydrophobic in nature.

Accordingly, we have studied hydrophobic compounds, isoprenoid ubiquinones (UQ8 and UQ10) and menaquinones (MK4 and MK7) (Scheme 4-2), to see if they show any adsorption peaks.

\textbf{Scheme 4-2.} Menaquinone (MK4) and Ubiquinone (UQ8)

The voltammetric comparison of the electrode depositing bacteria to the electrodes adsorbing these authentic quinone compounds led to the conclusion that the hydrophobic menaquinones and ubiquinones were the most likely candidates (Fig. 4-8). As can be seen in the figure, the peak potentials of the \textit{E. coli}-deposited electrode were virtually the same as those for the quinones at both pH values. Table 4-1 also lists the identical behavior among these electrodes. Along with this evidence, it was further confirmed that the evaporation of the
chloroform/methanol mixture that had been obtained by extraction from the *E. coli* cells gave a voltammogram with the mid-peak potentials identical to those for the *E. coli*-deposited electrode (Table 4-1). The extraction technique has been established for profiling of the bacterial quinones, UQn and MKn,\(^{26}\) and the identical potential values demonstrate that the peaks originated from the isoprenoid quinones.

**Figure 4-8.** Voltammetric responses of (A, D) *E. coli* (1.9 \(\times\) 10\(^9\) cells cm\(^{-2}\)), (B, E) MK4 and (C, F) UQ8 adsorbed on ITO electrodes at pH 7.0 and 3.5 and at 20 mV s\(^{-1}\). 10 \(\mu\)L of 1.0 \(\mu\)M MK4 and 20 nM UQ8 dissolved in EtOH-water (1:9 v/v) solutions were applied on the ITO electrodes. The short lines indicate the potentials of the bacterial peaks.

A more detailed inspection of Table 4-1 and Fig. 4-8 revealed that there were the same trends for not only the peak potentials but also for the peak shape regarding the pH change; the peak widths and peak separations, \(\Delta E_p\), of all three electrodes at pH 7.0 were smaller than those at pH 3.5. Adsorption voltammetry of the isoprenoid ubiquinones has been extensively studied,\(^{27-33}\) and a report on UQ10 showed that its peak separation and peak widths decreased with an increase in the solution pH;\(^{33}\) the current results agree well with the previous study.

The pH shifts of the mid-peak potentials, \(\Delta E_{m1}/\Delta pH\) and \(\Delta E_{m2}/\Delta pH\) (37 and 46 mV), were substantially lower than the Nernstian value of 60 mV, which was expected for the 2-electron and 2-proton transfer system at 303 K (Table 4-1); the slow electron transfer rates on the ITO electrode presumably gave rise to the inconsistency due to the strong dependence of \(\Delta E_m\) on the transfer coefficient. Table 4-1 demonstrates that the shifts of the *E. coli*-deposited electrode agree with the values of the authentic UQ8 and MK4. Furthermore, the shift of UQ10 in the pH range between 4 and 7 was reported to be \(\sim\)40 mV,\(^ {33}\) which also agrees with the observed result herein (37 mV).
Table 4-1. Electrochemical characteristics of *E. coli*, menaquinones (MKn), and ubiquinones (UQn) at 20 mV s\(^{-1}\) at 303 K\(^{a)}\)

| Compound     | pH 3.5 | | pH 7.0 | | | | | | | | | | | |
|--------------|--------|---|---|---|---|---|---|---|---|---|---|---|---|
|              | \(E_{m,1}\) | \(E_{m,2}\) | \(\Delta E_{p,1}\) | \(\Delta E_{p,2}\) | \(E_{m,1}\) | \(E_{m,2}\) | \(\Delta E_{p,1}\) | \(\Delta E_{p,2}\) | \((\Delta E_{m,1}/\Delta pH)\) | \((\Delta E_{m,2}/\Delta pH)\) |
| *E. coli*    | 0.115  | -0.091 | 0.706 | 0.670 | -0.015 | -0.254 | 0.670 | 0.547 | 37 | 46 |
| *E. coli* extract\(^{a)}\) | 0.015 | -0.096 | 0.643 | -0.235 | 0.585 | 40 |
| MK4          | -0.096 | 0.643 | -0.235 | 0.585 | 40 |
| MK7          | -0.264 | 0.549 | 35 |
| UQ8          | 0.120  | 0.737 | -0.002 | 0.697 | 35 |
| UQ10         | -0.049 | 0.585 | 35 |

\(^{a)}\) Potential was measured against Ag|AgCl|sat. KCl. \(E_{m,1}\) and \(E_{m,2}\) are the arithmetic means of the peak potentials of c1 and a1, and of c2 and a2, respectively. \(E_{p,1}\) and \(\Delta E_{p,2}\) are the differences in the peak potential between c1 and a1 and between c2 and a2. \(\Delta E_{m,1}/\Delta pH\) and \(\Delta E_{m,2}/\Delta pH\) are the potential shifts of \(E_{m,1}\) and \(E_{m,2}\) against the unit pH change. \(^{b)}\) The quinones were extracted from *E. coli* with 2:1 chloroform/methanol mixture. A 10-µL volume of 9:1 water/the chloroform-methanol mixture was applied on a 4-mm ITO electrode.

As discussed above, the redox centers of the adsorbed compounds were safely identified as benzoquinone and naphthoquinone. However, the number of the prenyl-unit is difficult to determine by only electrochemical data; the \(E_{m}\) values similar between MK4 and MK7, and between UQ8 and UQ10, make the assignments difficult at the present stage (Table 4-1). Ma et al. reported that the cyclic voltammetric responses of UQn (n=1, 5, 10), each of which was inserted in a lipid bilayer structured on a gold electrode, gave rise to an increasing peak separation with an increase in the number of the prenyl unit.\(^{31,32}\) It is noteworthy that the shape and potentials of the voltammetric peaks for the c1/a1 couple are very similar to those for the reported UQ10 peaks, suggesting that the adsorbed ubiquinone would carry a relatively long isoprenoid chain.\(^{31}\) The length and degree of saturation of the side chain are often dependent on the growth conditions of a given species,\(^{34}\) and in bacteria, UQ8, UQ9, or UQ10 is usually the major component.\(^{26,35}\)

Two possible mechanisms for the emergence of the voltammetric peaks have been mentioned in the previous section; (1) bacteria excrete biofilms, which contain the quinones for extracellular electron transfer,\(^{22}\) and (2) the fractional breakdown of bacterial cells during dehydration leads to a release of the quinones, which have been inserted in the plasma membrane consisting of the lipid bilayer.
The first mechanism cannot be justified as the reason for the peak emergence in that the isoprenoid quinones are only slightly soluble in the hydrophilic biofilm or slime layer.\textsuperscript{36} In contrast, the hydrophobicity of the quinones well explains the second mechanism; the lipid bilayers (the quinone pool) fragmented on the destruction of the cells come into contact with the electrode surface to transfer the hydrophobic quinones onto the electrode surface (Scheme 4-1, B and C). The adsorption structure of UQn has been postulated as parallel to the electrode surface.\textsuperscript{29,37} Accordingly, UQn and MKn are adsorbed through the interaction between the electrode surface and the isoprenoid chain, after the hydrophobic structure of the lipid bilayer is impaired by desiccation and voltage stimulus.

The voltammetric peaks were also observed by other evaporation techniques; desiccation in a vacuum or by heat gave voltammograms similar to that in the desiccator, and the intensity ratio of the c1 peak was 1.0:1.2:1.4 for drying in a desiccator for 40 min at 295 K, drying with a heat gun for 1 min, and drying in a vacuum desiccator for 7 min at 294 K, respectively (n=2). The SEM images of heat-dried bacteria showed that the cytosols were exuded around the cells, and the viability estimated by the fluorescent dyes was nil (Fig. 4-4 D). However, the high fatality rate did not lead to a significant increase in the current. Interestingly, an \textit{E. coli} suspension (10 mL) microwaved for 30 s at 700 W did not give a sediment separable by centrifugation, but an application of this homogeneous liquid on an ITO electrode exhibited a voltammogram identical to that for the desiccator-dried electrode.

The peak current density of c1, \(i_p\), against the \textit{E. coli} density had a linear dependence up to \(8 \times 10^7\) cells cm\(^{-2}\) \((i_p = 79 + 6.0 \times 10^{-4} \times \text{cell density}; R^2=0.97)\) and the detection limit was \(7 \times 10^6\) cells cm\(^{-2}\) \((2.2 \times 10^5\) cells when applied on the 2-mm diameter electrode). Above this density range, the slope became less steep, and the current density leveled out to \(~2500\) nA cm\(^{-2}\) at the scan rate of 20 mV s\(^{-1}\); this dependence gave a linear plot when the logarithmic cell density was alternatively used \([i_p = -5.5 \times 10^{3} + 1.1 \times 10^5 \times \log \text{ (cell density)}; R^2=0.94]\). The percent standard deviations (100×s/average peak current) of the UQn and MKn peak heights were 12\% and 7\% for \textit{E. coli} and 14\% and 24\% for \textit{S. oneidensis} (n=7), respectively.

\textbf{4.3.4. Voltammograms of other bacteria}

Figure 4-9 show the voltammograms of several different bacterial species, i.e., \textit{E. coli}, pathogenic bacteria, redox bacteria photosynthetic bacteria, etc. The bacteria are classified as facultative anaerobic bacteria except for voltammograms (D) and (J), and as Gram-negative
bacteria except for (G), (J) and (K). All of these examples, except for *S. oneidensis*, show that the MK peaks were either smaller than the UQ peaks or undetectable. Menaquinones are assumed to be evolutionarily the most ancient type of isoprenoid quinones and to be associated with the reducing character of the atmosphere before the occurrence of oxygenic photosynthesis.\textsuperscript{34,35} Some bacteria have lost the ability to synthesize menaquinones, since menaquinols are readily oxidized by molecular oxygen to be unusable as electron and proton donors under aerobic conditions.\textsuperscript{35} The reciprocal relationship between UQn and MKn presumably gave rise to the smaller levels of MKn in aerobiosis. *S. oneidensis* (H), often used in MFCs, exhibited two pairs of distinctive peaks. Especially, this species produced MKn more than the others, suggesting the importance of this quinone to provide electrons to extracellular substances, such as metal oxides and MFC anodes (see Scheme 4-1A).\textsuperscript{16}

Figure 4-9. Voltammetric responses of the ITO electrodes prepared with (A) *Escherichia coli*, (B) *E. coli* (O157:H7), (C) *E. coli* (O157:HNM), (D) *Pseudomonas aeruginosa*, (E) *Vibrio parahaemolyticus*, (F) *Salmonella enterica*, (G) *Staphylococcus aureus*, (H) *Shewanella oneidensis*, (I) *Rhodobacter sphaeroides*, (J) *Bacillus subtilis var. natto*, (K) *Corynebacterium glutamicum*, and (L) coliform bacteria isolated from the prawn law meat. All recorded in the pH 7.0 phosphate buffer at a scan rate of 20 mV s\textsuperscript{-1} in the anoxic condition at 303 K. The bacteria used in these experiments were cultured overnight (~18 h).

Photosynthesizing bacteria, *R. sphaeroides* (I), only showed the UQn peaks, which might be partly assigned to structurally similar plastoquinone existing in the reaction center of the photosynthetic system.\textsuperscript{38,39} *Bacillus subtilis var. natto* (J) gave only weak peaks, and
C. glutamicum (K), which has been used for the industrial production of glutamic acid, exhibited a trace amount of MKn (not clearly seen in the voltammogram). Above the inner membrane, Gram-positive bacteria (G, J, and K) have a thick and stable peptidoglycan layer, which might have led to the lower peak currents. Coliform bacteria (L), the classification of which has been used as a bacterial indicator of sanitary quality of foods and water, are a group of bacteria commonly found in soil and surface water and typically of intestinal origin. The bacteria isolated from law prawn meat had three different species identified as Serratia spp., Klebsiella spp. and Cronobacter spp. by the PCR technique (Table 4-2). It should be noted that these independent species only exhibited two pairs of redox peaks all at the same potentials, as seen in (L). In conclusion, it is believed that the hydrophobic transfer of isoprenoid ubiquinones and menaquinones onto an ITO electrode easily proceeds for many species of Gram-positive and negative bacteria and can be used for profiling the quinone levels.

Table 4-2. Characterization of the coliform isolates from prawn

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Score/bit</th>
<th>Most probable bacterial spp.</th>
<th>No. of colonies</th>
<th>Identity/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>760</td>
<td><em>Escherichia coli</em></td>
<td>1/1</td>
<td>100</td>
</tr>
<tr>
<td>Prawn meat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S_1_1</td>
<td>486</td>
<td><em>Serratia</em> spp.</td>
<td>1/12</td>
<td>97</td>
</tr>
<tr>
<td>S_1_2</td>
<td>185</td>
<td><em>Klebsiella</em> spp.</td>
<td>4/12</td>
<td>93</td>
</tr>
<tr>
<td>S_4_1</td>
<td>292</td>
<td><em>Klebsiella</em> spp.</td>
<td>3/12</td>
<td>94</td>
</tr>
<tr>
<td>S_6_4</td>
<td>749</td>
<td><em>Cronobacter</em> spp.</td>
<td>4/12</td>
<td>96</td>
</tr>
</tbody>
</table>

4.3.5. Redox mechanisms

It has been discussed above that the voltammetric peaks result from the adsorption of the isoprenoid quinones. A reason for this assignment is that the currents of the four peaks were linearly proportional to the scan rate (Fig. 4-5). In this section, the results of voltammetric simulation are discussed in order to obtain more mechanistic evidence for the adsorption. A simulation model based on the Frumkin-type isotherm was devised to explain the voltammograms consisting of the sharp cathodic and broad anodic peaks, which resulted from the interactions between adsorbed species. The simulation was made for *S. oneidensis* after the procedure published by Laviron. The low solubility of the isoprenoid ubiquinone and menaquinone in water allowed the presumption of simple surface-confined reactions. Hence, the reactions are expressed in terms
of eq. 1, and the differential equations for these reactions (eqs. 2A and 2B, given in APPENDIX) can be numerically solved:

\[
\begin{align*}
O_{1\text{ads}} + n_1e^- & \rightleftharpoons R_{1\text{ads}} \\
O_{2\text{ads}} + n_2e^- & \rightleftharpoons R_{2\text{ads}}
\end{align*}
\]
eq 1

Quinones can be assumed to take up two electrons to form quinols in an aqueous medium of neutral pH, i.e. \(n_1 = n_2 = 2\). The diffusion of protons from the solution was not explicitly formulated, because the simulation assumed a fixed pH. Figure 4-10 demonstrates that the simulation well reproduced the observed voltammogram using the parameters listed in the caption; the excellent agreement between the simulation and experiment supports the above reaction model.

A standard potential obtained by simulation is more accurate than a mid-peak potential \(E_m\), which is estimated from the arithmetic means of peak potentials, due to the strong dependence of \(E_m\) on the transfer coefficient, \(\alpha\), for slow electron transfer. The \(E^\circ\) values of ubiquinone and menaquinone are reported to be -0.086 and -0.274 V, which compare well to the values obtained by the simulation, -0.064, and -0.322 V, respectively. The differences in the potential between the experiment and simulation can result from the fact that the potential value obtained by the simulation is the surface standard potential, \(E^s\), which is a function of the adsorption coefficients of an oxidant \((b_O)\) and reductant \((b_R)\): \(E^s = E^\circ - (RT/nF) \ln (b_O/b_R)\). The presence of the isoprenoid chains might also affect the redox potentials of the quinones.

The simulation also gave accurate information on the surface density of the adsorbed molecules. The total density of \(1.8 \times 10^{-11} \text{ mol cm}^{-2}\) for both the adsorbed species suggests that they formed a monolayer close to saturation with small molecules. A study of the adsorption of UQ10 on a Hg electrode reported a maximum surface density of \((2.5 \pm 0.7) \times 10^{-11} \text{ mol cm}^{-2}\), which suggested that the density in this study was close to the maximum value. The value close to the maximum density suggested that the empty cells on the electrode (Fig. 4-4 C) did not strongly interact with the surface, thus allowing the quinones to spread all over the surface. This saturation also suggests that the lipids forming the cell walls do not have strong interactions with the surface to compete with the quinones, though some of them might be left there (Scheme 4-1C).
Figure 4-10. Voltammograms (–––) simulated and (•••) obtained by an experiment for S. oneidensis. Parameters used in the simulation are as follows:

\[ n_1 = n_2 = 2, T = 303 \text{ K}, v = 0.010 \text{ V s}^{-1}, A = 1.0 \text{ cm}^2, \Gamma_1 = 9.7 \times 10^{-12} \text{ mol cm}^{-2} \]
\[ \Gamma_2 = 7.8 \times 10^{-12} \text{ mol cm}^{-2}, E_{s1} = -0.064 \text{ V}, E_{s2} = -0.322 \text{ V} \]
\[ k_{s1} = 6.12 \times 10^{-5} \text{ s}^{-1}, k_{s2} = 2.22 \times 10^{-5} \text{ s}^{-1}; \alpha_1 = 0.62; \alpha_2 = 0.61 \]
\[ \beta_1 = 0.047, \gamma_1 = -\beta_1; \mu_1 = 0.47, \lambda_1 = -\mu_1; \beta_2 = 0.070; \gamma_2 = -\beta_2; \mu_2 = 0.46; \lambda_2 = -\mu_2 \]

(See APPENDIX for the definition of each parameter)

4.3.6. Dynamic profiles of ubiquinone and menaquinone depending on oxygen availability

Figure 4-11 shows the peak currents of UQn and MKn during the culture of E. coli. Bacterial samples taken during the culture profiled the sigmoidal OD curve, as expected (C). It is interesting to note here that MKn was produced more slowly than UQn (A). UQn and MKn perform reciprocal functions in E. coli; whereas, in general, UQn is primarily involved in aerobic respiration, MKn serves as an electron carrier during anaerobic respiration. As E. coli can control the UQ8 and MK8 production within minutes,\(^{46}\) they can flexibly change the production rates during culture. The delayed production of MKn in Fig. 4-11 (A) is probably due to the transition from an aerobic to anaerobic respiration, because the E. coli was batch-wise cultured in an airtight plastic tube; an E. coli suspension of 15 mL was cultured in a 50 mL tube.
Figure 4-11. Increase in the voltammetric peak currents during the cultivation of *E. coli*: (A) the current densities, and (B) Log (current density / OD$_{600}$) for the UQn, MKn and pMK peaks, and (C) OD$_{600}$ of the culture medium. The bacteria were cultured at 310 K, and voltammograms were recorded at 303 K at 20 mV s$^{-1}$ using the electrode with a 2 mm diameter.

In the middle of the culture, a small cathodic peak, denoted as pMK, appeared at -0.467 V between the UQn and MKn peaks (-0.364 and -0.556 V) as an intermediate (see Fig. 4-12). The pMK production preceding the MK synthesis suggests that the pMK species would be a precursor of MKn. It has been reported that demethylmenaquinone-8 (DMK8) is the parent molecule of MKn in the final methylation step.34 Whereas the DMK synthesis is initiated within 10 s after the shift to anaerobiosis, an increase in the MK content is much more gradual.46 As the production of MK slower than that of DMK is consistent with the pMK accumulation in the middle phase of the culture, DMK is the most likely candidate for the pMK species.

Figure 4-12. Cyclic voltammograms observed during cultivation of *E. coli* at 310K. The samples were taken at the cultivation time of (a) 3 h and (b) 7 h. Scan rate, 20 mV s$^{-1}$; electrode diameter, 2 mm. The potential was referred to the Ag|AgCl|sat. KCl electrode.

In these experiments, attempts were made to quench the conversion of DMK to MK by heat evaporation, which thermally deactivated the 2-DMK methyltransferase, the enzyme to add a methyl group to DMK. This heat evaporation increased the magnitude of the pMK peak by
more than twice that of the evaporation in the desiccator, suggesting again that the pMK peak can be assigned to DMK. The redox potential of DMK was reported to lie between UQ and MK ($E^{\circ'} = +112, +36,$ and $-74$ mV vs. NHE for UQ, DMK, and MK, respectively $^{47}$), which further agreed with the identification of pMK as DMK.

Figure 4-11 B indicates that the UQn content per *E. coli* cell during the first 7 h was higher than that for the stationary phase. Although the short culture time of less than 5 h can lead to a large evaluation error (±0.5 at 3h), the trend is likely, judged by extrapolation. During the lag phase of a culture, bacteria are not yet able to divide but are actively preparing chemical components necessary for growth, such as RNA, and enzymes. The UQn production would be one of such cases, and its active production led to the rise rate of the current vs. culture time curve slightly higher than that for OD even during the exponential phase. The MKn content showed an opposite trend, which could be explained by the pMK production occurring in parallel.

Figure 4-13 compares the UQ, pMK and MK productions under oxygen sufficient and insufficient culture conditions for two different types of bacteria. The culture made at 303 K, which was 7 degrees lower than for Fig. 4-11, allowed these bacteria still to develop in number and to produce pMK, when was sampled at 18 h. *P. aeruginosa*, which is an obligate aerobe, few grew in the oxygen deficient condition (B), as expected, and respiration constrained in aerobiosis made pMK and MKn unworthy of production, simply leading to their negligible syntheses (A). For *E. coli* (a facultative anaerobe), the anaerobic condition (D) proliferated the bacteria fewer than under the aerobic condition (C). However, the usefulness of naphthoquinol in the anaerobic condition up-regulated the production of MKn as well as pMK (D).

![Figure 4-13. Distribution of UQ, pMK and MK for (A and B) *P. aeruginosa*, (C and D) *E. coli*, cultured in (A and C) aerobic and (B and D) anaerobic conditions. All the bacteria were cultured at 303 K for 18 h. Scan rate, 20 mV s$^{-1}$](image_url)
The quinone levels in microorganisms have been analyzed by laborious procedures, which include the repeated sequence of extraction by an organic solvent and centrifugation, followed by a chromatographic measurement. In contrast, the present technique provides a much less laborious way concerning the sample preparation, in that one sample can be prepared in just 1 min by heat evaporation without using any organic solvents. In addition, a voltammetric measurement can be made in 5 min per sample, comparing to >20 min for the HPLC analysis.

A comparison was made between the UQn content in bacteria quantized by the present technique to that by the solvent extraction technique. The content of UQn for *E. coli* was reported to be 0.2–0.5 µmol g⁻¹ (dry mass), while a more recent study indicated 1.0–1.5 µmol g⁻¹. Using a dry cell weight of 150 fg cell⁻¹, the content from the peak area has been estimated to be 1.0 µmol g⁻¹ for the *E. coli* aerobically grown, which compares well to the above published values. The comparable value demonstrates that this technique can make high-throughput quinone profiling possible for microorganisms.

### 4.4. Conclusion

In this chapter, a novel bacterial detection technique by damaging bacterial cells through dehydration has been developed. Isoprenoid quinones in the cells were transferred onto an ITO electrode without using any organic solvents. As a result, two pairs of redox peaks were observed, assigned to surface-confined isoprenoid ubiquinone and menaquinone, which were both present in the quinone pool of the cells. It has also been revealed in this study that both Gram-positive and negative bacteria generate these well-defined peaks by the above treatment. This technique can be used for not only the detection of bacteria, but also profiling of the ubiquinone and menaquinone, the contents of which vary reflecting their living conditions.

In this respect, the present technique paves the way for the preparation of samples for quinone profiling, proven in this study to be much less laborious than the chemical extraction technique in that one sample can be prepared just in 1 min by heat evaporation of a target suspension.
APPENDIX

The surface reaction of adsorbed species 1 and 2 are expressed in terms of Eq. 1. The surface coverage ($\chi$) of each adsorbed species is defined by the following equations, using the surface densities for the oxidized form of species 1, $\Gamma_{O1}$, for the reduced form of species 1, $\Gamma_{R1}$, for the oxidized form of species 2, $\Gamma_{O2}$, and for the reduced form of species 2, $\Gamma_{R2}$.

$$
\chi_{O1} = \frac{\Gamma_{O1}}{\Gamma_{T1}}; \chi_{R1} = \frac{\Gamma_{R1}}{\Gamma_{T1}}; \chi_{O2} = \frac{\Gamma_{O2}}{\Gamma_{T2}}; \chi_{R2} = \frac{\Gamma_{R2}}{\Gamma_{T2}}
$$

The total surface concentrations, $\Gamma_{T1}$, and $\Gamma_{T2}$, are assumed to take constant values, and hence:

$$
\chi_{O1} + \chi_{R1} = 1; \chi_{O2} + \chi_{R2} = 1
$$

The observed voltammetric current is the sum of the currents, i.e. $i_1$ for adsorbed species 1, and $i_2$ for species 2, and is expressed by the following equations on the assumption that the number of electrons, $n$, is the same for both the redox species:

$$
i = i_1 + i_2 = nF\Lambda R_1 \frac{d\chi_{O1}}{dt} + \Gamma_{T1} \frac{d\chi_{R1}}{dt} + nF\Lambda R_2 \frac{d\chi_{O2}}{dt} + \Gamma_{T2} \frac{d\chi_{R2}}{dt} = \frac{n^2F^2AV}{RT} - \Gamma_{T1} \psi
$$

where the current function, $\psi$, is defined using the normalized potential, $\eta$, as follows:

$$
\psi = \frac{d\chi_{R1}}{d\eta} + g \frac{d\chi_{R2}}{d\eta}; \eta = \frac{nF}{RT} (E - E_{s,1}); g = \frac{\Gamma_{T2}}{\Gamma_{T1}}
$$

In the above equations, $E$ and $E_{s,1}$ denote the electrode potential and surface standard potential, respectively. The currents are also expressed by the following equations based on the Frumkin adsorption isotherm:

$$
i_1 = nFAK_{s,1} \Gamma_{T1} \left\{ \chi_{O1} \exp(-\alpha_{O1} \eta) \exp(-2\beta_1 \chi_{O1} - 2\gamma_1 \chi_{R1}) - \chi_{R1} \exp(\alpha_{O1} \eta) \exp(-2\lambda_1 \chi_{R1} - 2\mu_1 \chi_{O1}) \right\}
$$

$$
i_2 = nFAK_{s,2} \Gamma_{T1} \left\{ \chi_{O2} \exp(-\alpha_{O2} \eta) \exp(-2\beta_2 \chi_{O2} - 2\gamma_2 \chi_{R2}) - \chi_{R2} \exp(\alpha_{O2} \eta) \exp(-2\lambda_2 \chi_{R2} - 2\mu_2 \chi_{O2}) \right\}
$$

where $\beta_1$, $\beta_2$, $\gamma_1$, $\gamma_2$, $\lambda_1$, $\lambda_2$, $\mu_1$, and $\mu_2$ are the constants characterizing the Frumkin adsorption model, and $\alpha$'s are the transfer coefficients defined by:

$$
\alpha_{O1} + \chi_{O1} = 1; \alpha_{R1} + \chi_{R1} = 1
$$
The gradients of the coverages with respect to $\eta$ are then expressed as follows:

\[
\frac{d\chi_{R1}}{d\eta} = m_1 \left\{ \chi_{O1} \exp(-\alpha_{R1}\eta) \exp(-2\beta_{1}\chi_{O1} - 2\gamma_{1}\chi_{R1}) - \chi_{R1} \exp(\alpha_{O1}\eta) \exp(-2\lambda_{1}\chi_{R1} - 2\mu_{1}\chi_{O1}) \right\}
\]

\[
\frac{d\chi_{R2}}{d\eta} = m_2 \left\{ \chi_{O2} \exp(-\alpha_{R2}\eta) \exp(-2\beta_{2}\chi_{O2} - 2\gamma_{2}\chi_{R2}) - \chi_{R2} \exp(\alpha_{O2}\eta) \exp(-2\lambda_{2}\chi_{R2} - 2\mu_{2}\chi_{O2}) \right\}
\]

eq. 2A

eq. 2B

where the normalized electron transfer rate constants, $m_1$, and $m_2$, are defined by the following equations using the standard rate constants, $k_s,1, k_s,2$, and the scan rate, $v$:

\[
m_1 = \frac{RT}{nFA} \frac{k_{s,1}}{v} ; m_2 = \frac{RT}{nFA} \frac{k_{s,2}}{v}
\]

The differential equations, eqs. 2A and 2B are numerically solved to obtain the current function values as a function of $\eta$.\textsuperscript{43,51}
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CHAPTER V

Summary

In this study, various bacteria, including Gram-negative and Gram-positive ones were immobilized in polypyrrole and poly(3,4-ethylenedioxythiophene) (PEDOT) films. Thanks to negative charges located on their outer membrane, the insertion of bacteria into the polymer film becomes straightforward and automatically occurs, in order to compensate the positive charges appeared on polymer backbone during electrochemical deposition. After doping, bacteria remain active, allowing the communication through electrochemical analysis. Besides CPs, bacteria were also directly deposited on other material such as gold, glassy carbon, and indium-tin oxide coated glass, in order to obtain a hydrophobic transfer of quinones from bacterial cells to electrode surface, without using any solvent.

The purpose of this study is to develop electrochemical platforms for the observation of bacterial activity, using conducting polymers (CPs) as the inclusion matrices and using redox shuttle molecules transferred from bacteria to electrode surfaces. These devices work not only for the detection of bacteria, but also as platforms for monitoring bacterial activity.

Chapter 1 provides a brief introduction of bacteria, conducting polymers, isoprenoid quinones, and related studies.

Chapter 2 introduces the immobilization of various bacteria into the CP films by electrochemical deposition. The doping state of bacteria in the polymer films (PPy and PEDOT) varied according to the polymerization conditions. The viability of bacteria in the polymers their tolerance for attachment to various electrode materials was evaluated as well. In comparison with glassy carbon, gold or indium-tin oxide coated (ITO) glass, conducting polymers appeared as an appropriate material for entrapping bacteria in regard to their viability, density and distribution. As a result of respiration, bacteria doped on the polymer film showed a clear response to the addition of glucose as a nutrient, suggesting that this platform can serve as a simple but powerful analytical tool for bacterial activity observation.

In chapter 3 the electrochemical characteristics of polymer film doped with bacteria and bacterial viability are discussed. The sharp cathodic peaks and broad anodic peaks were observed in voltammogram of PEDOT/bacteria film, which are very similar to voltammetric responses of ubiquinones as inserted in a lipid bilayer structured on a gold electrode, suggest
that ubiquinones, excreted from bacteria originated the redox peaks. The results showed that both potential and time interval of polymerization, studied in this research have effect on viability of bacteria. In fact, thickness of polymer film determines this rate; more than 90% of bacteria found viable after polymerization at a film thickness of above 0.5 µm. Further investigation on stability of doped bacteria against electric stimulus revealed that they are relatively secure under anodic scans (from 0.0 V to +0.5 V) while the cathodic scans (between 0.0 V and -0.7 V) killed about 80% of *P.aeruginosa* and *S.oneidensis*. However, *E.coli* remained stable, even after 30 cycles of electrochemical scanning.

A rapid detection technique of bacteria by damaging bacterial cell walls through desiccation has been reported in chapter 4. By directly applying a bacterial suspension on an indium-tin oxide coated glass and evaporating, two pairs of well-defined redox peaks were observed in phosphate buffer pH 7, which were then assigned to isoprenoid ubiquinone (UQn) and menaquinone (MKn) present in bacterial cell envelopes. The hydrophobic transfer of quinones from various bacteria, including both Gram-negative and Gram-positive species to electrode surface once their membranes are damaged by heat or voltage stimulus suggests the possible detection of the quinones without using solvent. The dynamic profiles of UQn and MKn depending on oxygen availability were also evaluated, using this technique, indicating that it can be used not only for bacteria detection, but also for profiling of isoprenoid quinones, which play important roles in electron and proton transfer, in microorganisms.

Two different observation platforms for monitoring bacterial activity have been developed in these studies. In both systems, the observation was based on electrochemical methods, which offered potential analyzing tools and detecting devices for bacterial activity due to their simplicity and cost-effectiveness.
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## List of publications

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<td>Voltammetric Detection and Profiling of Isoprenoid Quinones Hydrophobically Transferred from Bacterial Cells</td>
<td>D.Q.Le A.Morishita S.Tokonami T.Nishino H.Shiigi M.Miyake T.Nagaoka</td>
<td>Analytical Chemistry, DOI:10.1021/acs.analchem.5b01772</td>
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