**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**BACTERIAL SYNTHESIS OF
NANOCATALYST AND BIOFILM-
TEMPLATED NANOCATALYTIC
SYSTEM FOR WATER
PURIFICATION**

NG CHUN KIAT

**INTERDISCIPLINARY GRADUATE
SCHOOL**

2017

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**A thesis submitted to the Nanyang Technological
University in fulfilment of the requirement for the
degree of Doctor of Philosophy**

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LIST OF ABBREVIATIONS

× g	g-force in centrifuge
°C	Degree Celsius
μg	Microgram
μL	Microlitre
μM	Micromolar concentration
μm	Micrometre
μmoles	Micromoles
2D	Two dimension
3D	Three dimension
AC	Activated carbon
Ag	Silver
C	Carbon
c-di-GMP	Bis-(3'-5')-cyclic dimeric guanosine monophosphate
CFU	Colony forming unit
CLSM	Confocal laser scanning microscopy
COM	Center of mass
Cr	Chromium
CTC	5-cyano-2,3-di-4-tolyl-tetrazolium chloride
Cu	Copper
DAPI	4'-6-diamidino-2-phenylindole
DGCs	Diguanylate cyclases
DMRB	Dissimilatory metal-reducing bacteria
DO	Dissolved oxygen
<i>E. coli</i>	<i>Escherichia coli</i>
e.g.	Example
E1	Estrone
E2	17β-estradiol

E3	Estriol
EAB	Electrically-active biofilm
EDCs	Endocrine disrupting chemicals
eDNA	Extracellular Deoxyribonucleic Acid
EDX	Energy-Dispersive X-Ray Spectroscopy
EE2	17 α -ethynylestradiol
EET	Extracellular electron transport
EPS	Extracellular polymeric substances
etc	et cetera
FDR	False Discovery Rate
Fe	Iron
Fe(III)-NTA	Ferric nitrilotriacetate
FHL	Formate-hydrogen lyase
GFP	Green fluorescent protein
h	Hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HFBA	Heptafluorobutyric acid
HPLC	High performance liquid chromatography
IC50	Half maximal inhibitory concentration
kDa	kilo-dalton
kV	kilo-volt
LB	Lysogeny Broth
MB	Methylene blue
MD	Molecular dynamics
metal(loid)	Metal and metalloid
min	Minute
mL	Milli-liter
MOPS	(3-(N-morpholino)propanesulfonic acid
MRI	Magnetic resonance imaging
mRNA	Messenger RNA

MS	Mass spectrometry
MTR	Metal reducing
MV	Methylviologen
N	Nitrogen
NaCl	Sodium chloride
nd	no data
Ni	Nickel
nm	Nanometre
NPs	Nanoparticles
O	Oxygen
OMCs	Outer membrane cytochromes
P	Phosphorous
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PCB	Polychlorobiphenyls
PCR	Polymerase chain reaction
Pd	Palladium
PDEs	Phosphodiesterases
PES	Polyethersulfone
PGA	Poly- β -1,6-GlcNAc
PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
pm	Picometre
PME	Particle mesh Ewald
PMF	Potential of mean force
PS	Polysulfone
PVDF	Poly(vinylidene fluoride)
qPCR	Quantitative polymerase chain reaction
RMSD	Root mean squared displacements
RNA	Ribonucleic acid
roGFP	Redox sensitive green fluorescent protein
ROS	Reactive oxygen species

RP	Reverse phase
RPKM	Reads assigned per kilo-base of target per million mapped read
rpm	Rounds per minute
<i>S. oneidensis</i>	<i>Shewanella oneidensis</i> MR-1
SCX	Strong cation exchange
SEM	Scanning Electron Microscopy
SMD	Steered molecular dynamics
SPC	Single point charge
SPR	Surface plasmon resonance
Tc	Technetium
TCE	Trichloroethylene
TCEP	Tris(2-carboxyethyl) phosphine
TEAB	Triethylammonium bicarbonate
TEM	Transmission Electron Microscopy
Ti	Titanium
TOF	Time-of-flight
U	Uranium
UF	Ultrafiltration
UV	Ultra-violet
v/v	Volume by volume
vol	Volume

LIST OF PUBLICATIONS

Journal papers

Ng CK, Bope DC, Nalapraju A, Cheng Y, Lu L, Wang R, Cao B (2017) Concentrating synthetic estrogen 17 α -ethinylestradiol using microporous polyethersulfone hollow-fiber membranes: Experimental exploration and molecular simulation. Chem Eng J **314**: 80-87.

Ng CK, Cao B (2015) What exactly are you filtering out? Environ Sci Technol **49 (9)**: 5259-5260.

Ng CK, Sivakumar K, Liu X, Madhaiyan M, Ji L, Yang L, Tang C, Song H, Kjelleberg S, Cao B (2013) Influence of outer membrane c-type cytochromes on particle size and activity of extracellular nanoparticles produced by *Shewanella oneidensis*. Biotechnol Bioeng **110(7)**: 1831-1837.

Ng CK, Tan TKC, Song H, Cao B (2013) Reductive Formation of Palladium Nanoparticles by *Shewanella oneidensis*: Role of Outer Membrane Cytochromes and Hydrogenases. RSC Adv **3(44)**: 22498-22503.

Liu T, Yu Y, Deng X, **Ng CK**, Cao B, Wang JY, Rice S, Kjelleberg S, Song H (2015) Enhanced *Shewanella* biofilm promotes bioelectricity generation. Biotechnol Bioeng **112**: 2051-2029.

Luhung I, Wu Y, **Ng CK**, Miller D, Cao B, Chang V (2015) Protocol improvement for low concentration of DNA-based bioaerosol sampling and analysis. PLoS One **10(11)**: e0141158.

Zhang Y, **Ng CK**, Cohen Y, Cao B (2014) Cell growth and protein expression of *Shewanella oneidensis* in biofilms and hydrogel-entrapped cultures. Mol Biosyst **10(5)**: 1035-1042.

Book Chapter

Ng CK, Mohanty A, Cao B (2015) Biofilms in Bio-Nanotechnology, in Bio-Nanoparticles: Biosynthesis and Sustainable Biotechnological Implications (ed O. V. Singh), John Wiley & Sons, Inc, Hoboken, NJ. doi: 10.1002/9781118677629.ch4

Conference presentations

Ng CK, Cao B (2016). Concentrating 17 α -Ethinyl Estradiol (EE2) Using PES Hollow-Fibre Membranes: Experimental Exploration and Molecular Modelling. 2016 AIChE Annual Meeting, San Francisco, CA, USA. (**Oral Presentation**)

Ng CK, Cao B (2016). Engineering Biofilm for Remediation of Environmental Chromium(VI) Contamination. 6th International Conference on Environment Science and Biotechnology, Kyoto, Japan. (**Oral Presentation**) [Won the Best Oral Presentation award]

Ng CK, Ding Y and Cao B (2014). Understanding and harnessing the power of biofilm matrix: What we have learned from *Shewanella* biofilms. Biofilm6, Vienna, Austria. (**Poster Presentation**)

ABSTRACT

Microorganisms have the potential to change the oxidation state of metals and these microbial processes have opened up a new window for novel applications including biosynthesis of metal nanomaterials. Planktonic cells based synthesis of metal nanomaterials has been studied previously; however, much of the mechanistic details are still relatively unknown. The application of planktonic cells-based metal nanomaterials synthesis is constrained to batch and fed-batch processes. Moreover, planktonic cells have low tolerance to toxic substrates or products which undermine the synthesis efficiency of metal nanomaterials.

Microbial biofilms might offer a solution. Biofilms are structured, surface-associated, microbial communities that are prevalent in most natural environments, engineering systems and clinical settings. One of the most important features of a biofilm is the presence of extracellular polymeric substances (EPS) that form a matrix and encase the cells in the biofilm. Cells in biofilms often form structurally stable communities and show a remarkable resistance against various biocides. Hence, we hypothesize that biofilm may be a good candidate to use for the synthesis of metal nanomaterials.

This project aims to develop a robust and efficient biofilm-based nano-catalytic system which is applicable to water purification and environmental remediation. To understand the mechanism of nanomaterial production by microorganism, we used model organism *Shewanella oneidensis* MR-1 to synthesize silver, silver sulfide and palladium nanoparticles. We showed that the synthesized metal and metalloid nanoparticles exhibit catalytic activity. Microbial synthesis of silver and silver sulfide is influenced by outer membrane cytochromes, while synthesis of palladium nanoparticles (NPs) is influenced by periplasmic [NiFe]-hydrogenase. In addition, we

also revealed that in extracellular biosynthesis of NPs, the usually neglected non-cell-associated NPs could have high catalytic activity, highlighting the need of novel methods that can efficiently retain extracellular NPs in the biosynthesis processes.

The second part of the project focused on exploring the reductive formation of Pd(0) nanoparticles in biofilms under ambient conditions, understanding the metabolic responses of the biofilms during the process of palladium nanoparticles synthesis, and developing a robust nanocatalytic biofilm through genetic engineering. We showed that the *S. oneidensis* biofilms can reduce Pd(II) and accumulate Pd(0) nanocrystals with a size of 10-20 nm in the biofilm matrix and in the cell membrane even under bulk aerobic conditions, and that the *S. oneidensis* biofilms with Pd(0) nanocrystals exhibit nanocatalytic activity. However, we also observed that Pd(II) exposure inhibits cellular respiration and energy metabolism of *S. oneidensis* cells, posing a risk of biofilm detachment.

To improve the robustness of biofilm-based nanocatalyst, we explored two approaches: (1) genetic manipulation via c-di-GMP pathway and (2) physical alteration via controlled pyrolysis. In previous studies, c-di-GMP has been reported to be able to influence the production of EPS and influence biofilm processes such as cell dispersal. In our study, we showed that we can increase the production of EPS and alleviate the biofilm detachment during the biofilm's exposure to Pd(II). The insertion of plasmid pYedQ2 can increase the production of cytoplasmic c-di-GMP, an upstream signalling molecule which influences several key cellular processes, such as cellular motility, extracellular electron transport, iron uptake and biofilm formation in *S. oneidensis* MR-1.

Further, we also showed that controlled pyrolysis leads to carbonisation of biofilm and formation of stable, abiotic heteroatom (N and P)-doped carbon-palladium (C-Pd) nanocomposite catalyst. Moreover, the pyrolysed biofilm-templated C-Pd nanocomposite catalyst show high Cr(VI) reduction, and maintained high reduction rate even on the 5th catalytic cycle. Although the pyrolysed heteroatom-doped carbon on its own has no Cr(VI) reduction activity, it seems to enhance the catalytic activity of the Pd nanocrystals in the C-Pd nanocomposite.

CHAPTER 1: INTRODUCTION

1.1. Background

Microorganisms can change the oxidation state of metals and these microbial processes open up new opportunities for researchers to explore novel applications, for example, the biosynthesis of metal nanomaterials (De Corte, Hennebel et al. 2012, Sintubin, Verstraete et al. 2012). In contrast to conventional chemical and physical methods that often involve the use of toxic chemicals and harsh conditions, microbial synthesis of nanomaterials can be achieved in aqueous phase under gentle and environmentally benign conditions (Lloyd, Byrne et al. 2011). This approach has become an attractive focus in current green nanotechnology research towards sustainable development.

Certain biogenic metal nanomaterials such as palladium, silver, and gold nanoparticles exhibit catalytic activities and the bacteria-nanomaterial associations, known as metalised bacterial cells, can be exploited for environmental remediation (De Windt, Boon et al. 2006, Hennebel, De Gusseme et al. 2009, Forrez, Carballa et al. 2011) and synthesis of fine chemicals (Sobjerg, Gauthier et al. 2009, Heugebaert, De Corte et al. 2012, Bennett, Mikheenko et al. 2013). The metalised cells reported in most, if not all, previous studies were prepared using planktonic cultures. The planktonic cells based approach is restricted to batch and fed-batch bioprocesses which is of lower efficiency compared to continuous reactor operations. Moreover, additional steps are required to recover the metallised planktonic cells which are often time-consuming and energy intensive. Another limitation of using planktonic cells is that they have low tolerance to metal and metalloid ions which might compromise the integrity of the bacteria cells during the biosynthesis of metal nanomaterials.

Biofilms are microbial communities where the cells are encased and immobilised by a self-fabricated extracellular polymeric substances (EPS) matrix (Flemming and Wingender 2010). Unlike planktonic cells, biofilm cells have much higher tolerance to antimicrobial agents and other toxic chemicals (Flemming 2011). It is also comparatively much simpler to recover the immobilised biofilm cells as compared to the free floating planktonic cells. Biofilm-nanomaterial associations are suitable catalyst for both batch and continuous reactor operations. Hence, the use of biofilms to mediate the synthesis of biogenic nanocatalysts is a promising approach to developing catalytic systems for environmental and chemical applications.

1.2. Research gaps and challenges

Biofilms may be as effective as planktonic cells for the synthesis of biogenic nanomaterials, while providing added advantages such as heightened tolerance to toxicity, ease of nanomaterial recovery, and versatility in operational modes. However, various limitations prevent the biofilm-based synthesis of nanomaterials and its subsequent deployment of biofilm-nanomaterials associations for practical applications to be widely carried out.

The mechanisms of nanomaterial synthesis using planktonic and biofilm cells are not well understood. Moreover, the role of EPS matrix in production of nanomaterials within biofilm is largely unexplored. Since the synthesis of metal nanomaterials involves reduction of metal ions, it may be possible that certain cells surface and EPS matrix-associated redox proteins, especially those with extracellular electron transport (EET) capabilities, can contribute to the synthesis of nanomaterials within biofilm (Cao, Shi et al. 2011). It is important to elucidate and understand the mechanistic details of

the biofilm-based synthesis of nanomaterials for its optimisation and execution in large-scale industrial settings.

The use of biofilm for the synthesis of nanomaterials may affect how cells grow and function. As some metal ions are toxic to planktonic cells (Melber, Keller et al. 2002), certain cells responses (e.g. cell dispersal) may be triggered due to stress when exposed to the metal ions used for nanomaterial synthesis. Furthermore, natural biofilm development involves stages such as biofilm detachment. If not controlled properly, biofilm detachment and cell dispersal can lead to loss of precious nanomaterials.

Biofilm engineering can be deployed to alleviate the bottlenecks that limit the widespread use of biofilm-based bioprocesses, such as synthesis of biogenic nanomaterials. Understanding the underlying mechanisms of nanomaterial synthesis and engineering biofilm to be robust with lower detachment are key challenges associated with biofilm-based synthesis of nanomaterial for practical applications.

1.3. Objectives

The overall objective of this research is to explore the use of biofilms for the synthesis of nanocatalysts and the development of biofilm-templated nanocatalytic systems. Specifically, the following three aims are to be achieved:

- (i) To explore the role of key bacterial membrane and matrix-associated redox proteins, such as c-type cytochromes and hydrogenases, in biosynthesis of nanocatalysts.
- (ii) To engineer biofilms to make it more robust with lower detachment.

(iii) To remove biomass from nanocatalytic biofilms through controlled pyrolysis to achieve and maintain a higher catalytic activity.

1.4. Scope

This thesis contains seven chapters, namely introduction, literature review, four research chapters and conclusion with future recommendations.

In Chapter 1, a brief background on microbial synthesis of nanomaterials and biofilm-based biocatalysis is introduced. This chapter discusses the advantages of biofilm-based synthesis of nanomaterials as compared to planktonic approaches, while listing the limitations of such biofilm-based bioprocesses. This chapter also describes the objective and scope of this study in detail.

Chapter 2 entails the literature review for the entire thesis. This chapter examines the bacterial synthesis of nanomaterial and its application in environmental remediation. This is followed by the introduction of biofilm mode of life, biofilm matrix components and biofilm biology. It also describes the significance of bioremediation as compared to other current technologies in the removal of emerging contaminants from the environment.

Chapter 3 presents the mechanistic study on the role of outer membrane cytochromes and hydrogenases of *Shewanella oneidensis* MR-1 on the reductive formation of biogenic silver and silver sulfide nanoparticles.

Chapter 4 reports the mechanistic study on the role of outer membrane cytochromes and hydrogenases of *S. oneidensis* MR-1 on the reductive formation of biogenic palladium nanoparticles.

Chapter 5 presents the in situ bioreductive formation of palladium nanoparticles in the biofilm matrix and engineering of biofilms with nanocatalytic activity (a.k.a nanocatalytic biofilm) via genetic manipulation of bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) level.

Chapter 6 presents the engineering of nanocatalytic biofilm via controlled pyrolysis to form robust biofilm-templated heteroatom-doped carbon-palladium nanocomposite catalyst.

Chapter 7 concludes with the summary of studies covered in this thesis and provides some future recommendations.

CHAPTER 2: LITERATURE REVIEW

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2.1. Background on bacterial synthesis of nanomaterials

There are many instances where nature acts as a guide to human advancement in technology. In the search for a more environmentally benign approach to synthesize nanomaterials, researchers drew inspiration from the way microorganisms interact with metals and metalloids in the natural environment. Microorganisms have the potential to change the oxidation state of metals and metalloids via oxidation and reduction, and these microbial processes (i.e. biomineralisation) have provided access to novel applications including biosynthesis of metal and metalloid nanomaterials (Hennebel, De Gusseme et al. 2009, Durán and Seabra 2012).

Conventional synthesis of metal nanomaterials uses chemical and physical methods that often involve the use of toxic chemicals and harsh conditions (Raveendran, Fu et al. 2003). In contrast, microbial synthesis of metal nanomaterials can be achieved in aqueous phase under gentle and environmentally benign conditions (Hennebel, De Gusseme et al. 2009, Lloyd, Byrne et al. 2011). This approach has become an attractive focus in current research on green nanotechnology research towards sustainable development.

Many types of microbes can be utilised to catalyse the synthesis of biogenic metal or metalloid nanomaterials (De Corte, Hennebel et al. 2012, Sintubin, Verstraete et al. 2012). Biogenic metal nanomaterials, such as that of gold, silver and palladium, can exhibit catalytic properties which are applicable in fine chemical synthesis and other industrial catalytic processes (Deplanche, Merroun et al. 2012, Corbos, Ellis et al. 2013). Table 2.1 summarises the recent studies on bacterial synthesis of gold, silver and palladium nanomaterials.

Table 2.1. Summary of recent studies on bacterial synthesis of gold, silver and palladium nanomaterials.

Metal	Bacteria	Size (nm)	Electron Donor(s)	Application(s) of synthesized nanomaterial	References
Gold	<i>Shewanella oneidensis</i>	5 - 20	Hydrogen gas, formate	No data (nd)	(De Corte, Hennebel et al. 2011)
		5 - 30	Yeast extract	nd	(Suresh, Pelletier et al. 2011)
	<i>Rhodopseudomonas capsulata</i>	10 - 20	Pyruvate	nd	(He, Guo et al. 2007)
	Mixed Community (Anaerobic Sludge)	15 - 21	Acetate	Catalysis for hydrogen production	(Khan, Lee et al. 2013)

	<i>Shewanella algae</i>	10 - 20	Lactate	nd	(Konishi, Tsukiyama et al. 2007)
	<i>Geobacillus stearothermophilus</i>	5 - 14	Casein, soybean meal, dextrose	nd	(Mohammed Fayaz, Girilal et al. 2011)
Silver	<i>Bacillus licheniformis</i>	40 - 60	Nitrate	nd	(Kalimuthu, Suresh Babu et al. 2008)
	<i>Geobacillus stearothermophilus</i>	5 - 35	Casein, soybean meal, dextrose	nd	(Mohammed Fayaz, Girilal et al. 2011)
	<i>Lactobacillus farciminis</i>	14 - 20	Tryptic soy	Antimicrobial activity	(Sintubin, De Windt et al. 2009)
	<i>Lactobacillus rhamnosus</i>	13 - 18		Antimicrobial activity	

	<i>Lactobacillus plantarum</i>	16 - 22		Antimicrobial activity	
	<i>Lactobacillus fermentum</i>	5 - 15	Tryptic Soy	Virus disinfection in water	(De Gusseme, Hennebel et al. 2011)
	<i>Shewanella oneidensis</i>	2 - 6	Yeast extract	Antimicrobial activity	(Suresh, Pelletier et al. 2010)
		40 - 42	Lactate	Antimicrobial activity	(Ng, Sivakumar et al. 2013)
	<i>Bacillus cereus</i>	5	Yeast extract	Antimicrobial activity	(Sunkar and Nachiyar 2012)
	<i>Ureibacillus thermosphaericus</i>	1 - 100	Yeast extract	nd	(Juibari, Abbasalizadeh et al. 2011)

	<i>Corynebacterium</i>	5 - 15	Beef extract	nd	(Zhang, Li et al. 2005)
	<i>Brevibacterium casei</i>	10 - 50	Beef extract	Anti-coagulative activity	(Kalishwaralal, Deepak et al. 2010)
	<i>Staphylococcus aureus</i>	160 - 180	Beef extract	Antimicrobial activity	(Nanda and Saravanan 2009)
	<i>Rhodobacter Sphaeroides</i>	2 - 16	Malate	nd	(Bai, Yang et al. 2011)
	<i>Plectonema boryanum</i>	1 - 50	Nitrate	nd	(Lengke, Fleet et al. 2007)
	<i>Geobacter sulfurreducens</i>	20 - 40	Acetate	nd	(Law, Ansari et al. 2008)

Palladium	<i>Geobacter sulfurreducens</i>	14 - 36	Hydrogen gas, acetate	nd	(Yates, Cusick et al. 2013)
	<i>Shewanella oneidensis</i>	45 - 55	Hydrogen gas, formate	Reductive degradation of perchlorate	(De Windt, Boon et al. 2006)
		10 - 20	Formate	nd	(Ng, Cai Tan et al. 2013)
	<i>Desulfovibrio desulfuricans</i>	45 - 55	Hydrogen gas, formate	Hydrogen generation from hypophosphite	(Yong, Rowson et al. 2002)
	<i>Clostridium pasteurianum</i>	8 - 16	Biogenic hydrogen gas	Immobilisation of chromate	(Chidambaram, Hennebel et al. 2010)
	<i>Pseudomonas aeruginosa</i>	16 - 28	nd	nd	(Srivastava and Constanti 2012)

	<i>Rhodobacter sphaeroides</i>	3 - 10	Hydrogen gas, formate	Dechlorination of chlorinated aromatic compounds	(Redwood, Deplanche et al. 2008)
	<i>Clostridium butyricum</i>	10 - 50	Biogenic hydrogen gas	Dehalogenation of diatrizoate	(Hennebel, Van Nevel et al. 2011)
	<i>Citrobacter braakii</i>	10 - 30			
	<i>Klebsiella pneumoniae</i>	nd			
	<i>Enterococcus faecium</i>	nd			
	<i>Escherichia coli</i>	nd			
	<i>Bacteroides vulgatus</i>	nd			

	Cupriavidus necator	10 - 30	Hydrogen gas	Catalysis for Suzuki–Miyaura and Mizoroki–Heck reactions	(Gauthier, Søbjerg et al. 2010)
	Cupriavidus metallidurans	50 - 150			

Various bacteria, mostly dissimilatory metal reducing bacteria, have been employed to produce metal(loid) nanomaterials (Lovley 1991). Among them, metal-reducing bacteria from *Shewanella* and *Geobacter* genera are of great interest because they have a relatively well-characterized electron transport chain that can transport electrons across cell membranes and is capable of reducing metal(loid)s at the cell exterior, giving a relatively narrow size distribution and minimizing post-production extraction and purification (Shi, Richardson et al. 2009, Sintubin, Verstraete et al. 2012). Strict anaerobic conditions are often required for *Geobacter* species, while *Shewanella* can grow well in both aerobic and anaerobic conditions, rendering *Shewanella* an important bacterium in bio-nanotechnology. *S. oneidensis* is one often-used metal-reducing bacterium in biosynthesis of metal(loid) nanomaterials.

Although a wide range of bacteria have been used for the synthesis of metal(loid) nanomaterials, the molecular mechanisms underlying the biosynthesis processes are largely unknown. Based on previous literature, the conclusions we can draw include: (1) both cell-mediated and biologically influenced redox reactions are often involved; (2) dominating mechanisms vary in different bacteria; and (3) primary molecular sites responsible for the biosynthesis in a certain bacterium can be very different for different metal(loid)s.

In the biosynthesis of nanomaterials by bacteria, both cell-mediated and biologically influenced reactions have been reported. In organisms such as *E. coli* and *P. aeruginosa*, the conversion of soluble metal(loid)s to their insoluble nanosized structures is a general detoxification mechanism where surface proteins, polysaccharides, and periplasmic proteins are often involved and there are no specific biomolecular sites responsible for the biosynthesis of metal(loid) nanomaterials.

In *S. oneidensis*, c-type cytochromes, a class of redox proteins, have been shown to play a central role in metal reduction (Fredrickson, Romine et al. 2008). The outer membrane of *S. oneidensis* contains c-type cytochromes including two best-characterized outer membrane c-type cytochromes (OMCs) MtrC and OmcA (Shi, Chen et al. 2006). Figure 2.1 shows the illustration of the metal-reducing (MTR) pathway in *S. oneidensis*. Extensive studies have suggested the important role of OMCs in efficient reduction of various metal(loid)s including Fe(III), Cr(VI), Tc(VII), and U(VI) (Marshall, Beliaev et al. 2006, Marshall, Plymale et al. 2008, Belchik, Kennedy et al. 2011).

Figure 2.1. Illustration of the metal-reducing (MTR) pathway in *S. oneidensis*. The MTR pathway consists of a number of electron carrier proteins including CymA, MtrA, MtrC, and OmcA. The electrons obtained from bacterial metabolism are transferred across the periplasmic space to the outer membrane, where MtrC and OmcA can use the electrons to reduce metal(loid)s. Reproduced from (Ng et al 2015) with permission from “John Wiley and Sons”

In addition to c-type cytochromes, hydrogenases comprise another class of redox proteins that play a central role in microbial reduction of metals in *S. oneidensis*. Figure 2.2 shows the model of *S. oneidensis* MR-1 [NiFe]-hydrogenase in Tc(VII) reduction.

Figure 2.2. Model of *S. oneidensis* MR-1 [NiFe]-hydrogenase-mediated Tc(VII)O₄⁻ reduction. [NiFe]-hydrogenase, situated in the periplasm, catalyse the reduction of Tc(VII) by coupling the oxidation of H₂ into H⁺. Reproduced from (Shi, Belchik et al. 2011) with permission from “American Society for Microbiology”.

The genome of *S. oneidensis* MR-1 encodes both a [FeFe]-hydrogenase HydAB and a [NiFe]-hydrogenase HyaAB. HydA functions as a hydrogen-forming hydrogenase, while HyaB can function either in formation or oxidation of hydrogen as a bidirectional hydrogenase. Previous studies have shown that the [NiFe]-hydrogenase of MR-1 was involved in H₂-driven reduction of Tc(VII) to Tc(IV)/Tc(V) (Marshall, Plymale et al. 2008, Shi, Belchik et al. 2011). However, the contribution of OMCs and hydrogenases to bioreduction of silver and palladium in *S. oneidensis* has not been well elucidated.

The gap in the detailed understanding of bioreduction mechanism, along with other limitations, hampers the widespread use of bacteria for the synthesis of nanomaterials.

Currently, most of the biogenic nanomaterials were prepared by planktonic cell cultures. While the planktonic cells-based approach for the synthesis of nanomaterials works well in the laboratory-scale studies (De Corte, Hennebel et al. 2012), this method is only applicable to batch or fed-batch processes and not suitable for highly efficient continuous processes commonly deployed in industrial settings which uses less down-time for maintenance (Gross, Hauer et al. 2007). Moreover, it is energy intensive and time consuming to harvest the metal nanomaterials that are attached onto planktonic cells. Another limitation of planktonic cells is their low tolerance to metal and metalloid ions, which affects the integrity of the cell walls and viability of the planktonic cells, and prevents the use of higher metal ion concentration for faster rate of nanomaterial synthesis.

As such, there is a need to explore a new system for biosynthesis of nanomaterials, where cells can be immobilised for easy recovery of the produced metal nanomaterials and where cells have higher tolerance to toxic metal ions for faster rate of metal nanomaterial synthesis. Cells in biofilm mode seem to be a promising approach to synthesise nanomaterials as biofilm cells are able to retain the functionality of planktonic cells while compensating their limitations.

2.2. Biofilm and biofilm-based processes

2.2.1. Biofilm matrix

Biofilm mode of life is ubiquitous in most natural and man-made environments. Bacteria switch between the planktonic and biofilm mode of life depending on the environmental stresses such as salinity, availability of nutrient and predation etc. Unlike in planktonic mode where individual cells suspend freely in the liquid medium, a biofilm consist of bacteria communities encased and immobilised by self-produced

extracellular polymeric substances (EPS) matrix and are found between interfaces, namely solid-liquid interfaces (e.g. river-bed biofilms), solid-air interfaces (e.g. bacteria colonies on agar plate) and liquid-air interfaces (e.g. pellicles) respectively (Branda, Vik et al. 2005). There are also other types of microbial communities such as floc, granule and sludge which are not attached to an interface but shared many characteristics of biofilms.

With increasing knowledge on biofilm, it became apparent that besides their diverse physical appearances, biofilms which constitute various compositions of bacteria species can exhibit strikingly different chemical and biological processes. These biofilm processes include, but not limited to, photosynthesis, denitrification, methanogenesis, bioluminescence, nitrogen fixation, fermentation and metal reduction, all of which are important to biogeochemical cycling and other processes in nature.

One of the most important features of a biofilm is the presence of extracellular polymeric substances (EPS) which form a three dimensional matrix and encase the bacteria within the biofilm. Extensive studies have demonstrated a wide range of advantages of EPS matrix for the biofilm mode of life (Flemming and Wingender 2010, Flemming 2011). The EPS matrix allows bacteria to form structurally stable biofilm communities and show a remarkable tolerance against various toxic compounds and antibacterial agents. Other beneficial characteristics of biofilm include resistance against desiccation, attachment to favourable environment and the obstruction of grazing by predators etc.

The components of EPS in biofilms are very complex and differ from species to species, but generally consist of biomolecules such as carbohydrate, proteins, lipids

and extracellular DNA (eDNA) (Flemming and Wingender 2010, Flemming 2011). Figure 2.3 illustrates the schematic diagram of biofilm and biofilm matrix with EPS components.

Figure 2.3. The EPS matrix at different dimensions. a) Model of a surface-attached biofilm. b) Major components in the EPS matrix, such as polysaccharides, proteins and extracellular DNA. c) Possible interaction that contribute to the overall structure integrity of the EPS matrix. d) Molecular modelling simulation of the interaction between alginate (right) and lipase (left) of *Pseudomonas aeruginosa* in aqueous solution. Reproduced from (Flemming and Wingender 2010) with permission from “Nature Publishing Group”

The complexity of EPS and their spatial and temporal heterogeneity within biofilms poses great difficulty in isolating and analysing them. Several EPS extraction techniques have been reported (Jahn and Nielsen 1995, Wuertz, Spaeth et al. 2001, Liu and Fang 2002, Comte, Guibaud et al. 2006, Adav and Lee 2008), and these shed light on the identity of some EPS components. As of now, several biomolecules which influence biofilm development have been identified. Many strains of *Pseudomonas spp.* have the capacity to excrete at least three types of extracellular polysaccharides, namely alginate, Pel and Psl, and these are important to the structural stability of the biofilms formed (Kolter and Greenberg 2006, Ryder, Byrd et al. 2007). Poly- β -1,6-GlcNAc (PGA) and curli are found to affect the development of biofilm by promoting abiotic surface binding, intercellular adhesion and mediate cohesion in EPS matrix of *Escherichia coli* biofilm (Prigent-Combaret, Prensier et al. 2000, Wang, Preston et al. 2004, Ryu and Beuchat 2005). In *Bacillus subtilis* biofilms, amyloid fibers were shown to support the structure of the biofilm by promoting cell-cell binding and surface adhesion (Romero, Aguilar et al. 2010). In many species of bacteria such as *Rhodobacter sphaeroides* and *Vibrio cholerae*, flagella play an important role not only in motility, but contribute to biofilm stability as well (Pratt and Kolter 1998, Prigent - Combaret, Prensier et al. 2000, Watnick, Lauriano et al. 2001, Branda, Vik et al. 2005, Wilkinson, Chacko et al. 2011). In *Shewanella spp.*, a 285 kDa multi-domain protein BpfA has been associated with the function of promoting biofilm formation (Theunissen, De Smet et al. 2010, Cao, Shi et al. 2011).

2.2.2. Biofilm Formation and Development

Biofilm development can be described in various stages where cells switch from the free-swimming planktonic mode to the sessile biofilm mode. Figure 2.4 illustrates the

various stages of biofilm development. The first stage of biofilm development starts with the initial attachment of planktonic cells onto a substratum, usually in an environment that is favourable for cell growth (Halan, Buehler et al. 2012). The attached cells then produce EPS which holds the cells firmly to the substratum. Besides EPS, outer cell membrane appendages such as flagellum and pili form structural anchorage and contribute to cell attachment onto substratum (Flemming and Wingender 2010, Flemming 2011). Intermolecular forces such as hydrophobic interactions, hydrogen bondings, electrostatic interactions and Van der Waals forces within EPS help to promote cell adhesion onto substratum.

Figure 2.4. Main stages of biofilm development cycle – initial attachment, microcolony formation, biofilm maturation, and biofilm dispersal.

The second stage of biofilm development begins with cell multiplication and increase secretion of EPS to form microcolonies on the substratum. The EPS serves as a scaffold and provide structural support and stability to the microcolonies. Moreover,

the EPS matrix functions as external barrier and provides resistance against toxic substances, antibacterial agents and predation from grazers (Flemming 2011).

In the third stage of biofilm development, the microcolonies grow in size and form 'mushroom-shaped' towering structures with high surface to volume ratio to better facilitate mass transfer of substrates and waste products into and out of the biofilm matrix (Bishop, Zhang et al. 1995, O'Toole and Kolter 1998). Microenvironments exist heterogeneously in the mature biofilm due to diffusion limitation. For example, oxygen level is high near the surface of the biofilm, but drastically declines in regions deep within the biofilm that houses respiring cells (Zhang, Ng et al. 2014). The EPS can also serve as food source for the cells during periods of low nutrient level, and prevents desiccation due to its water retention capability (Flemming and Wingender 2010, Flemming 2011).

In the fourth stage of biofilm development, mature biofilm starts to disperse with environmental cues such as low nutrient level, oxygen depletion, low level of nitric oxide and changes in temperature and availability of iron (McDougald, Rice et al. 2012). At this stage, cells regain motility and escape from the EPS matrix in search for another conducive environment for growth and colonisation, and thus, the biofilm development cycle repeats over and over again (Landini, Antoniani et al. 2010).

Biofilm development is a complicated process and it requires delicate regulation systems to coordinate at each developmental stage. It is essential for regulatory systems such as intracellular signalling (e.g. cyclic-di-GMP) and intercellular signalling (e.g. quorum sensing) to work in tandem for successful execution of and smooth transition between various stages of biofilm development (Landini, Antoniani et al. 2010).

2.2.3. Biofilm Regulation and Signalling

Bacterial cells were long thought to be 'individualistic', where the decision-making processes are solely influenced by the organism itself. However, in 1979, Nealson and Hastings observed bioluminescence in *Vibrio fischeri*, and that such phenomenon occurred only when there is high cell density. This is currently considered the model system for quorum sensing in most gram-negative bacterium. Quorum sensing is an intercellular signalling process where bacteria detect and alter behaviour based on the extracellular concentration of secreted signal molecules. The concentration of the signal molecules, also known as autoinducers, is typically proportional to the cell density and a minimum level or threshold autoinducer concentration must be reached before a specific behaviour, caused by autoinducer-dependent gene expression, can take place.

Quorum sensing has been associated to many microbial processes, including but not limited to bioluminescence, biofilm development, virulence, and siderophore production. Techniques which alters autoinducers to influence biofilm formation have been relatively well explored, but are mainly focused on inhibiting biofilm maturation and generally limited to applications in pharmaceuticals and anti-biofouling (Prouty, Schwesinger et al. 2002, Hammer and Bassler 2003, Hentzer and Givskov 2003, Salta, Wharton et al. 2010). Such techniques can be broadly classified in two categories: autoinducer degradation and autoinducer receptor inhibition. Recent application in anti-biofouling based on autoinducer degradation describes the use of an enzyme-immobilised nanofiltration membrane to mitigate biofouling based on quorum quenching, where the immobilised enzyme, acylase, previously established to degrade autoinducer acyl-homoserine lactone (AHL) effectively, suppresses the

production of EPS, and hence prevents biofilm maturation (Kim, Choi et al. 2011). On the other hand, for application in pharmaceuticals via autoinducer receptor inhibition, a recent study examined the effectiveness of various synthetic quorum sensing inhibitors and found that meta-bromo-thiolactone not only prevents virulence factor expression and biofilm formation but also protects *Caenorhabditis elegans* and human A549 lung epithelial cells from quorum-sensing-mediated killing by *Pseudomonas aeruginosa* (O'Loughlin, Miller et al. 2013).

Besides intercellular signalling, intracellular signalling is also an important component in biofilm formation and development. Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP), an important intracellular signalling molecule, acts as a global regulator which influences the switching between the planktonic and biofilm mode in bacteria cells. Figure 2.5 shows the structure and physiological function of c-di-GMP. C-di-GMP is synthesized by diguanylate cyclases (DGCs) and is broken down by phosphodiesterases (PDEs). DGC activity is influenced by GGDEF domain, while PDE activity is influenced by EAL domains. C-di-GMP is known to stimulate the synthesis of adhesins and other EPS matrix components, while inhibiting various forms of motility (Jenal and Malone 2006, Wolfe and Visick 2008, Hengge 2009). There are numerous studies on the manipulation of c-di-GMP to influence biofilm development, and this is done mainly through the genetic manipulation by inserting genes coding either the GGDEF or EAL domain to increase or decrease the level of c-di-GMP respectively (Simm, Morr et al. 2004, Gjermansen, Ragas et al. 2006, Kuchma, Brothers et al. 2007, Rakshe, Leff et al. 2011).

Figure 2.5. Structure and physiological function of c-di-GMP. Reproduced from (Hengge 2009) with permission from “Nature Reviews Microbiology”.

The influence of c-di-GMP on *S. oneidensis* MR-1 biofilm is of particular interest as MR-1 is a metabolically versatile organism with great relevance in environmental remediation and bionanotechnology. Moreover, computational analysis predicts that the *S. oneidensis* MR-1 genome contains 51 diguanylate cyclases, 27 phosphodiesterases, and 20 hybrid diguanylate cyclase or phosphodiesterase proteins, which places *S. oneidensis* MR-1 into a group of sequenced microorganisms with the highest normalised number of c-di-GMP signalling proteins (Fredrickson, Romine et al. 2008).

2.2.4. Biofilm Heterogeneity and Chemical Gradients

The structure of biofilms is very much influenced by physical stress in the environment. Biofilms tend to be more compact and thinner under high hydrodynamic shear (Liu

and Tay 2002). Moreover, various unique structural forms of biofilms, such as streamers, can be observed under different hydrodynamic conditions (Stoodley, Lewandowski et al. 1998). The structure of biofilms is said to be uneven, where the thickness, porosity and roughness differs at various sections of the biofilm matrix (Wimpenny, Manz et al. 2000). It is indeed this unevenness, or spatial heterogeneity, that allows various micro-environments to exist within biofilms which lies in seemingly homogeneous macro-environments.

There are many contributing factors to the spatial heterogeneity of biofilms, but it is unanimously agreed upon that chemical gradient, also known as diffusion gradient, plays a major role in orchestrating the formation of micro-environments within biofilms (Stewart and Franklin 2008). There are many reports on the chemical gradients within biofilm, and are mostly focused on metabolic substrates, intermediates and products. It is well documented that oxygen, a metabolic substrate, exhibits gradient of concentrations within biofilms. Table 2.2. shows the types of dissolved oxygen (DO) sensors used in biofilm studies, their advantages and disadvantages.

The oxygen gradient is resulted mainly from oxygen intake by actively respiring cells, where rate of oxygen consumption by respiration outstrip the rate of oxygen diffusion within the EPS matrix. This phenomenon produces micro-anoxic environments within biofilms which can accommodate strict anaerobes even under bulk aerobic conditions (Zhang, Ng et al. 2014). Similarly, the heterogenic distribution of metabolic products throughout biofilms also influences individual cell functions and cell-cell interactions, which may result in differentiated biological processes occurring at various depths and microenvironment within the biofilms.

Table 2.2. Dissolved oxygen (DO) sensors used in biofilm studies.

Type of sensor	Advantages	Disadvantages	Reference
Micro-electrode	Able to perform Spatial and temporal measurements in 3D.	Invasive and destructive.	(Nguyen, Cao et al. 2012)
Micro-optode	Able to provide 2D maps of DO concentrations in contact with the micro-optode.	Unable to sense DO within biofilms that is not in contact with micro-optode.	(Holst, Glud et al. 1997)
Fluorescent redox probe	Able to perform high resolution spatial measurements in 3D.	Not suitable for temporal measurements. Low sensitivity.	(Rodriguez, Phipps et al. 1992)
Microfluidic chip	Able to provide 2D maps of DO concentrations in contact with the chip.	Unable to sense DO within biofilms that is not in contact with the chip.	(Skolimowski, Nielsen et al. 2010)

2.2.5. Redox Activities of EPS

Reduction and oxidation (redox) activities play an important role in extracellular processes for both planktonic or biofilm mode. One such process is extracellular electron transport (EET) by dissimilatory metal-reducing bacteria (DMRB), where electrons can be transported out of the cell to extracellular electron acceptors during respiration. Extracellular electron transport is facilitated by redox shuttles, such as flavins (Marsili, Baron et al. 2008, Von Canstein, Ogawa et al. 2008), phenazines (Hernandez, Kappler et al. 2004, Rabaey, Boon et al. 2005), humic acids (Lovley 1991, Lovley, Coates et al. 1996, Milliken and May 2007), and by EET chains such as MTR respiratory pathway (Shi, Squier et al. 2007, Fredrickson, Romine et al. 2008, Shi, Richardson et al. 2009, Richardson, Butt et al. 2012).

EPS may play a role to immobilise these electron shuttles close to the cells within the matrix, so as to extend the redox capability of the cells. Using 5-cyano-2,3-di-4-tolyl-tetrazolium chloride (CTC) as fluorescent redox sensor, it was found that EPS extracted from sludge samples exhibits redox activities, where such activities are postulated to facilitate transformation of organics matters for cellular metabolic uses (Wuertz, Pfeleiderer et al. 1998). In another study, it was shown that EPS extracted from biofilm of environmental isolate *Shewanella spp.* contain at least 20 redox active proteins, where it is suggested that the EPS matrix forms a network of redox active proteins to facilitate extracellular electron transport between the cells and extracellular compounds within the matrix (Cao, Shi et al. 2011). Table 2.3 shows the recent studies on redox activity of biofilm.

Table 2.3. Recent studies on redox activity of biofilm.

Organism(s)	Reactor	Key findings	Reference
<i>Pseudomonas aeruginosa</i>, <i>Aspergillus fumigatus</i>	Agar plates	In coculture biofilms, <i>Pseudomonas aeruginosa</i> phenazine-derived metabolites can influence the development of fungal <i>Aspergillus fumigatus</i> by inducing asexual sporulation from initial weak vegetative growth.	(Zheng, Kim et al. 2015)
<i>Geobacter sulfurreducens</i>	Interdigitated microelectrode array	Redox gradient exists within <i>Geobacter sulfurreducens</i> biofilm, in which the concentration of electrons residing within the proposed redox cofactor network increases with increasing distance from the anode surface.	(Okamoto, Hashimoto et al. 2012)
<i>Pseudomonas aeruginosa</i>	Working electrode array	Integrated circuit-based electrochemical sensing platform, featuring an array of working electrodes and parallel potentiostat channels, can be used for the spatially resolved detection of phenazine produced by biofilm, and allows <i>in-situ</i> study of redox activities within biofilms.	(Bellin, Sakhtah et al. 2014)

<i>Shewanella oneidensis</i>	96-well plates	Redox sensitive fluorescence protein roGFP can be displayed onto cell surface of <i>S. oneidensis</i> by fusing it to the C-terminus of BpfA, a large surface protein. The surface displayed roGFP can be used as a sensor to quantify the extracellular redox status in the matrix of <i>S. oneidensis</i> biofilms, which is a novel approach to non-destructive monitoring of extracellular redox status in microenvironments within biofilms.	(Sivakumar, Mukherjee et al. 2014)
<i>Pseudomonas aeruginosa</i>	Bioelectrochemical cell	Different strains of <i>Pseudomonas aeruginosa</i> exhibit different phenazine-dependent electroactivity, depending on the substrates used. <i>Pseudomonas sp.</i> KRP1, isolated from a microbial fuel cell enrichment, produced high electroactivity, while PA01 showed very low phenazine production and electrochemical activity. The difference may reflect their adaptability in different environments.	(Bosire, Blank et al. 2016)

Besides electron shuttles, recent studies have attributed the contribution of bacterial nanowires for long-distance extracellular electron transfer for *Shewanella* and *Geobacter* that may facilitate energy distribution within biofilms (Gorby, Yanina et al. 2006, Reguera, Nevin et al. 2006). Bacterial nanowires are extracellular appendages that exhibit electrical conductivity. In *S. oneidensis*, it was found that mutant lacking outer membrane c-type cytochrome MtrC and OmcA do not produce nanowires that are conductive (El-Naggar, Wanger et al. 2010). Moreover, it was demonstrated that *S. oneidensis* MR-1 nanowires are cell membrane extensions of the extracellular electron transport components, such as MtrC and OmcA (Pirbadian, Barchinger et al. 2014). On the other hand, the nanowires of *G. sulfurreducens* are unilateral pili which are conductive regardless of the presence or absence of outer membrane c-type cytochrome (Reguera, Nevin et al. 2006).

The mechanism of electrical conductivity of bacterial nanowires is hotly debated, and two main models of nanowire's electron conductivity were established - multistep electron hopping and metal-like electron conduction. Evidences of multistep hopping of electrons are based on *S. oneidensis* studies, where outer membrane cytochromes MtrC and OmcA are found on the surface of nanowires and are directly responsible for the extracellular electron transfer along the nanowire (Pirbadian, Barchinger et al. 2014). In *G. sulfurreducens*, nanowires are comprised of pili that have metal-like electron conductivity attributed to overlapping pi-pi orbitals of aromatic amino acids (Malvankar and Lovley 2014).

The discovery of electron shuttles in EPS and bacterial nanowire furthers our knowledge on extracellular redox activity of bacteria and provides insights to possible electron

transfer mechanisms within the biofilm matrix. Further investigation is required to understand the mechanistic details of redox activity and electron conductivity within biofilm in order to fine tune and optimise the use of biofilms for industrial catalytic processes or environmental remediation purposes.

2.3. Immobilisation of nanoparticles in biofilm matrix

The synthesis of biogenic metal nanomaterials using planktonic bacteria is rather well studied (Liu, Gorby et al. 2002, Hennebel, De Gusseme et al. 2009, Lloyd, Byrne et al. 2011, Durán and Seabra 2012, Sintubin, Verstraete et al. 2012). On the other hand, there are not much research on the use of biofilm to synthesize metal nanomaterials, even though it is potentially a more effective and efficient option as compared to the use of planktonic cells, as discussed in previous chapters. This is not surprising as the bulk of the biofilm research revolves around eradicating and controlling the growth of biofilm for medical and antifouling applications (Singh, Schaefer et al. 2000, Simões, Simões et al. 2010, Arciola, Campoccia et al. 2012, Burmølle, Ren et al. 2014). To be fair, there is a substantial amount of research on understanding and optimising the removal of nutrient from waste water by naturally occurring biofilm communities in the form of flocculants and granules (Dangcong, Bernet et al. 1999, Wuertz, Bishop et al. 2003, McSwain, Irvine et al. 2005), but not much studies were done to engineer the biofilms for other applications e.g. synthesis of metal nanomaterials. In this last section of the review, literature related to the use of biofilm to synthesize or immobilise nanomaterials will be discussed in detail.

In the first study, *Serratia* biofilm, grown on the polyurethane foam within in a continuous air-lift fermenter, was used to immobilise Pd nanoparticles reduced by hydrogen gas

(Beauregard, Yong et al. 2010). The resultant biofilm-Pd was used as a catalyst to reduce soluble hexavalent chromium ion, Cr(VI), to insoluble trivalent chromium ion, Cr(III). Magnetic resonance imaging (MRI) technique was used to assess the Cr(VI) reduction as MRI is non-invasive and that Cr(III) is paramagnetic, which can be measured by magnetic resonance. Figure 2.6 shows the spatial and temporal magnetic resonance map of Cr(III) concentration from batch-mode reduction of Cr(VI) using biofilm-Pd catalyst.

Figure 2.6. Spatial and temporal magnetic resonance map of Cr(III) concentration from batch-mode reduction of Cr(VI) using biofilm-Pd catalyst. Concentration of Cr(III) decrease due to precipitation of solid Cr(OH)_3 and building up of gas bubbles (black regions within the reactor). Reproduced from (Beauregard, Yong et al. 2010) with permission from “John Wiley and Sons”.

However, it is important to note that the synthesis of Pd nanoparticles is not biofilm-mediated. Hydrogen gas was used to reduce Pd(II) ions to Pd nanoparticles, while the biofilm act as a solid substrate or scaffold to immobilise the nanoparticles. The biofilm in this study is not directly involved in the Pd reduction process, where the Pd(II) ions were chemically reduced and not biologically reduced to form Pd nanoparticles. Many different types of scaffold, such as cellulose and amine-functionalised beads, can be used to immobilise metal nanoparticles that are synthesized by chemical reduction (De Corte, Bechstein et al. 2012, Baruah, Das et al. 2015). This study did not investigate the

contribution of biofilm towards the reduction of Pd(II) and Cr(VI) beyond its role as a structural support. It did, however, showed that MRI is a great tool to measure and visualise spatial and temporal distribution of paramagnetic compounds within biological samples in a non-invasive manner.

In the second study, biofilms from anaerobic sludge are used to reduce silver ions, Ag(I), to silver (Ag) nanoparticles in TiO₂ aqueous suspension (Khan, Ansari et al. 2013). The Ag nanoparticles were precipitated with TiO₂ to form Ag@TiO₂ nanocomposites. The nanocomposites were used in photodecomposition of methylene blue (MB) in an aqueous solution under visible light irradiation, and the authors found that the nanocomposites showed much higher activity as compared to commercial TiO₂ nanoparticles. For the reduction of Ag(II), the authors did controlled experiments and found that both the anaerobic sludge (biofilm) and sodium acetate, presumably a carbon source and electron donor, are required for the reaction to occur. This shows that the reduction of Ag(II) is a biofilm-mediated process, where the cells in biofilm used sodium acetate as electron donor and Ag(I) as electron acceptor to achieve anaerobic respiration. Figure 2.7 shows the proposed mechanism for the EAB-mediated synthesis of Ag@TiO₂ nanocomposites.

Figure 2.7. Proposed mechanism for the EAB-mediated synthesis of Ag@TiO₂ nanocomposites. Reproduced from (Khan, Ansari et al. 2013) with permission from “The Royal Society of Chemistry”.

One interesting observation is that the Ag nanoparticles were found to precipitate onto TiO₂ in the bulk solution. According to the proposed mechanism, the electron transfer occurred primarily on the surface of the biofilm, and TiO₂ facilitated the electron transfer from the biofilm to the Ag(I) for the reduction to occur. This may be possible due to the man-made anaerobic condition by purging the reactor with nitrogen gas. However, one would expect that the biofilm can transfer electrons directly to Ag(I) and that the Ag nanoparticles can be attached onto the surface or within the biofilm, instead of requiring TiO₂ to act as an electron shuttle and that Ag nanoparticles are being attached onto the TiO₂ in the bulk solution. It has been well-established that biofilms are able to directly facilitate electron transfer on its surface and within the biofilm (Templeton, Trainor et al. 2001, Richardson, Edwards et al. 2012, Shi, Dong et al. 2016). Unfortunately, the authors did not include any details regarding the state of biofilm after Ag reduction, nor the

efficiency of the Ag reduction to support its proposed mechanism for Ag reduction. One possible explanation for the presence of Ag@TiO₂ is that the Ag reduction may have occurred both within the biofilm and on the surface of biofilm, and that the authors may have neglected to account for the Ag nanoparticles that are deposited on the biofilm.

In both studies, the metal reduction process is done after purging nitrogen gas in order to create a man-made anaerobic condition within the reactor. The rationale is that metal reduction by planktonic cells is usually inhibited by oxygen and requires bulk anoxic conditions to occur (Lloyd, Byrne et al. 2011, Durán and Seabra 2012). As discussed in previous section, the heterogeneity nature of biofilms, coupled with an oxygen diffusion gradient, allows for anoxic microenvironments within biofilms under bulk oxic condition. It is uncertain if such anoxic microenvironment within biofilms can support bioreduction of metals. There are limited studies on the biofilm-mediated synthesis of metal nanomaterials and the biological mechanism of metal bioreduction by biofilm is poorly understood. More studies are needed to elucidate the mechanistic details of biofilm-mediated synthesis of nanomaterials to improve the feasibility of biofilm-based bioprocesses to be used in applications such as industrial fine-chemical synthesis and environmental remediation.

CHAPTER 3: INFLUENCE OF OUTER MEMBRANE C-TYPE CYTOCHROMES ON PARTICLE SIZE AND ACTIVITY OF EXTRACELLULAR NANOPARTICLES PRODUCED BY *SHEWANELLA ONEIDENSIS*

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3.1. Introduction

Microorganisms have the potential to change the oxidation state of metals and these microbial processes have opened up a new window for novel applications including biosynthesis of metal nanomaterials (Gadd 2010, Lloyd, Byrne et al. 2011). Traditional approaches for the synthesis of metal nanomaterials, such as chemical, thermal, photochemical, sonochemical, electrochemical reduction, and ligand displacement and condensation of metal vapor, usually require harsh conditions and the use of toxic reagents and solvents (Dahl, Maddux et al. 2007). In contrast, microbial processes can synthesize nanomaterials in water under gentle and environmentally benign conditions. Hence, biological processes for the production of nanomaterials have become one of the attractive research focuses in current green nanotechnology towards sustainable development (Niemeyer 2001, Mandal, Bolander et al. 2006, Hennebel, De Gusseme et al. 2009, Durán, Marcato et al. 2011).

A wide variety of microorganisms including bacteria, fungi, and yeast have been exploited to synthesize metal nanoparticles (NPs) (Li, Chung et al. 2003, Bigall, Reitzig et al. 2008, Suresh, Pelletier et al. 2010, Jain, Bhargava et al. 2011, K, R et al. 2011, Kumar and Mamidyala 2011, Jung, Park et al. 2012). Some microorganisms are capable of producing NPs extracellularly, giving relatively narrow size distribution and minimizing post-production extraction and purification (Narayanan and Sakthivel 2010, Kalathil, Lee et al. 2011, Sintubin, Verstraete et al. 2012). Among these microorganisms, *Shewanella oneidensis* has attracted great interest because it is able to reduce a diverse range of metal ions and the extracellular electron transport chains responsible for transferring electrons across cell membranes have been relatively well characterized (Shi, Squier et al. 2007, Fredrickson, Romine et al. 2008, Shi, Richardson et al. 2009, Richardson, Edwards et al. 2012, Shi, Rosso et al. 2012). In *S. oneidensis*, the c-type cytochromes comprise a class of redox proteins that play a central role in metal reduction (Fredrickson, Romine et al. 2008). A large percentage of the membrane-bound c-type cytochromes were found to be associated with the outer membrane, including two best characterized outer membrane c-type cytochromes (OMCs) MtrC and OmcA (Shi, Chen et al. 2006). The most intriguing feature of OMCs is that they were also found to be associated with extracellular polymeric substances (EPS) secreted by *S. oneidensis* and contributed to extracellular formation of uranite NPs during U(VI) reduction (Marshall, Beliaev et al. 2006, Cao, Ahmed et al. 2011, Cao, Shi et al. 2011). Extensive studies have suggested an important role of OMCs in reduction of various metal(loid)s. However, how OMCs influence the particle size of extracellular NPs and their properties including antimicrobial, toxicological, catalytic, and optical features remains unknown.

The objective of the current study was to investigate the influence of OMCs, that is, MtrC and OmcA, on the size and activity of the extracellular NPs using the production of silver NPs (AgNPs) and silver sulfide NPs (Ag₂S NPs) by *S. oneidensis* MR-1 as a model system. We used MR-1 cell suspensions of the wild-type (WT) and the mutant lacking OMCs to reduce AgNO₃ or AgNO₃ and Na₂S₂O₃ to generate AgNPs or Ag₂S NPs, respectively. Particle size of the extracellular NPs from the WT and the mutant was compared. Our results show that, comparing with the WT, the mutant lacking OMCs produced significantly smaller NPs. We further characterized the activity of the AgNPs and Ag₂S NPs. AgNPs have been demonstrated to exhibit biocidal activity towards a broad range of bacteria and have been termed as a new-generation of antimicrobial (Morones, Elechiguerra et al. 2005, Sintubin, Verstraete et al. 2012); thus, we characterized the influence of OMCs on the antibacterial activity of the biogenic AgNPs. Ag₂S NPs have a documented catalytic property in reducing methylviologen (MV²⁺) (Kryukov, Stroyuk et al. 2004); hence, to investigate the impact of OMCs on the catalytic activity of the biogenic Ag₂S NPs, MV²⁺ reduction was used as a model catalytic reaction. Our results showed that the AgNPs and Ag₂S NPs produced by the mutant exhibited higher antibacterial activity and catalytic activity, respectively.

3.2. Materials and Methods

3.2.1. Bacterial Strains and Growth Conditions

S. oneidensis MR-1 WT and a mutant strain lacking MtrC and OmcA, that is, $\Delta(mtrC-omcA)$ were kindly provided by Dr. Liang Shi and Dr. Jim K. Fredrickson from Pacific Northwest National Laboratory, USA. Stock cultures were maintained in LB medium with

20% glycerol at -80°C . The cells were grown in LB medium containing 10 mM ferric nitrilotriacetate (Fe(III)-NTA) anaerobically for 16 h at 30°C with shaking at 200 rpm.

3.2.2. Extracellular Synthesis of Nanoparticles

After 16 h incubation, cells were harvested through centrifugation at $6,000 \times g$ for 10 min, washed twice with 50 mM MOPS buffer (pH 7.2) and re-suspended in MOPS buffer to a final cell density of ca. 1.2×10^9 cells/mL containing (i) 10 mM sodium lactate and 1 mM AgNO_3 (for AgNPs), or (ii) 10 mM sodium lactate, 1 mM AgNO_3 , and 1 mM $\text{Na}_2\text{S}_2\text{O}_3$ (for Ag_2S NPs). Each cell suspension was flushed with N_2 gas for 5 min and then incubated at 30°C on a rotary shaker (~ 200 rpm). After 120 h, cell suspensions were centrifuged at $10,000 \times g$ for 20 min to remove cells and the supernatants were collected (Cao, Shi et al. 2011). UV–visible spectra (600–360 nm) of the supernatants were obtained. Extracellular NPs in the supernatant were further harvested by using ultracentrifugation at $100,000 \times g$ for 30 min, washed three times with MilliQ water and dried at 50°C under vacuum.

3.2.3. Field Emission Scanning Electron Microscopy and Energy-Dispersive X-Ray Spectroscopy

Cell pellets were washed twice and then re-suspended in MilliQ water. A portion of each suspension was dried under vacuum and then coated with platinum using a vacuum electric sputter coater (JEOL JFC-1300; JEOL Asia Pte Ltd., Singapore). SEM images were then taken using a field emission scanning electron microscope (FE-SEM, JSM-7600; JEOL Asia Pte Ltd.) at a voltage of 2.0–5.0 kV. EDX spectrum was obtained using

an energy-dispersive X-ray spectroscope (AZtecEnergy, Oxford Instruments, Oxfordshire, UK).

3.2.4 Transmission Electron Microscopy

About 3 μ L of the as-synthesized biogenic AgNPs from each supernatant was applied to a carbon-coated copper grid and dried in the air for transmission electron microscope (TEM) imaging using a Jeol JEM-1230 TEM instrument operated at an accelerating voltage of 120 kV.

3.2.5 Size Distribution

The hydrodynamic diameters of the extracellular AgNPs and Ag₂S NPs were determined using Zetasizer (Nano ZS, Malvern Instruments, Worcestershire, UK). In order to make sure that the extracellular NPs were uniformly dispersed, 15 min ultrasonication in an S450H Ultrasonic Bath (50/60 Hz, 2000 W; Fisher Scientific, Singapore) was employed before each measurement.

3.2.6 Antibacterial Activity of Biogenic AgNPs

3.2.6.1 Minimal Inhibitory Concentration (MIC) Test

The MIC, defined as the lowest concentration of a certain compound that inhibits the growth of an organism. Specifically, *Escherichia coli* (ATCC 8739) was used for the MIC test and the bacterial cultures in LB media containing varying concentration of the biogenic AgNPs were incubated for 12 h. The minimal concentration at which no obvious cell growth could be observed ($OD_{600} < 0.1$) was considered as the MIC.

3.2.6.2 Bactericidal Assay

E. coli was used as a model organism to evaluate the toxicity of the biogenic AgNPs. LB cultures were prepared on a rotary shaker (~200 rpm) at 37°C and cells were harvested at the mid-exponential growth phase (after ~12 h incubation). The harvested cells were washed twice and then resuspended in 0.9% NaCl (final cell density ~10⁸ cells/mL). Bactericidal activity was tested in 96-well plates. Each well of the testing plate contained 100 µL of the biogenic AgNPs suspension (final concentration of 0 to 10 mg/L) and 100 µL of cell suspension. Plates were incubated at 37°C for 24 h. Cell viability was evaluated using a live/dead baclight bacterial viability kit (Molecular Probes, Eugene, OR). The percentage of live cells in each well was estimated based on green/red fluorescence ratio. Half maximal inhibitory concentration (IC₅₀) value, corresponding to the NPs concentration at which only 50% cells remain live after 24 h incubation, was calculated based on viability versus NPs concentration curves.

3.2.7 Inductively Coupled Plasma Mass Spectrometry

To determine the dissolution of Ag⁺ from the biogenic AgNPs, the suspension of 100 µg/mL AgNPs in 0.9% NaCl was filtered through a centrifugal filter unit (molecular weight cutoff 3 kDa; Amicon Ultra-4 3K). The total Ag⁺ concentration was quantified using an inductively coupled plasma mass spectrometer (ICP-MS; ELAN DRC-e; Perkin Elmer, Waltham, MA) as described previously (Suresh, Pelletier et al. 2010).

3.2.8 Catalytic Activity of Biogenic Ag₂S NPs

Both cell-associated Ag₂S NPs and supernatant Ag₂S NPs obtained from cell suspension were tested for catalytic activity in the reduction of methylviologen (MV²⁺) to a cation-radical MV^{•+} (λ_{max} = 605 nm and molar extinction coefficient ϵ_{605} = 13,700 M⁻¹ cm⁻¹). About 13 mg cell pellet (cells and cell-associated Ag₂S NPs; ~1 mg dried biomass) or 360 μ g of dried extracellular Ag₂S NPs (harvested from the supernatant) was resuspended in 450 μ L of MilliQ water, which was then mixed with 500 μ L of 20 mM Na₂S and 50 μ L of 20 mM MV²⁺. Upon addition of MV²⁺, optical density at wavelength of 605 nm (OD₆₀₅) was monitored continuously (1 s interval) for 5 min.

3.3 Results and Discussion

3.3.1 Biogenic AgNPs and Ag₂S NPs

Extracellular biosynthesis of AgNPs and Ag₂S NPs using cells of *S. oneidensis* MR-1 WT has been reported (Suresh, Pelletier et al. 2010, Suresh, Pelletier et al. 2011), where the biogenic NPs have been extensively characterized. In our experiments, SEM-EDX confirmed the presence of AgNPs and Ag₂S NPs, respectively (data not shown). Here, in this study, we found that the strain lacking OMCs, that is, $\Delta(mtrC-omcA)$ also produced extracellular AgNPs and Ag₂S NPs as indicated by a characteristic surface plasmon resonance (SPR) band at 410 nm in the UV–visible spectra (Mulvaney 1996, Magudapathy, Gangopadhyay et al. 2001, Suresh, Pelletier et al. 2010, Suresh, Pelletier et al. 2011) but with much lower SPR intensity, suggesting that the amount of extracellular NPs synthesized was significantly lower than using WT. The abiotic controls (buffer containing no cells) did not show the SPR absorbance band, confirming that the NPs

obtained were biogenic. These results implied an important role of OMCs in extracellular Ag(I) reduction, which is also supported by previous observation of the formation of nanoscale Ag(0) deposits on cell surface during Ag(I) reduction by *S. oneidensis* (Wang, Law et al. 2010). Although both the WT and the OMCs mutant could generate extracellular NPs, the question is whether these biogenic NPs differ in terms of particle size and activity or not? To address it, we characterized the particle size and their activities of the extracellular NPs produced by WT and the mutant.

3.3.2 Particle Size

TEM analyses (Fig. 1A–D) revealed that the extracellular biogenic NPs from WT and the OMCs mutant were generally spherical in shape and significantly smaller particles were obtained from the mutant. Consistent with the TEM observations, quantitative analysis of the particle size distribution for the NPs from both strains showed that extracellular NPs produced by the OMCs mutant $\Delta(mtrC-omcA)$ were significantly smaller than those produced by WT (24.4 ± 0.8 nm vs. 40.9 ± 1.0 nm for AgNPs and 27.6 ± 6.4 vs. 53.4 ± 12.4 for Ag₂S NPs, respectively) (Figure 3.1).

Figure 3.1. TEM images of the extracellular AgNPs (A and B), Ag₂S NPs (C and D) produced by different *S. oneidensis* strains: WT (A and C), $\Delta(mtrC-omcA)$ (B and D), and the average (n = 3) hydrodynamic diameters of the biogenic NPs (E).

Since many physicochemical and toxicological properties of NPs are size-dependent (Pal, Tak et al. 2007, Jiang, KimBetty et al. 2008, Park, Lim et al. 2011, Søbjerger, Lindhardt et al. 2011, Perez-Alonso, McCarthy et al. 2012), controlling the particle size in biosynthesis of NPs is critically important, which remains one of the main challenges. It has been reported that, by choosing different microbial systems, the desirable size of the NPs produced could be obtained (Sintubin, Verstraete et al. 2012). However, this strategy is not suitable for scalable biotechnological processes, where frequent change of workhorse is not sustainable. It would be ideal if the size of the extracellular NPs produced in microbial processes can be controlled through genetic manipulations of the microorganisms used for the synthesis. Here, our results show that the lack of OMCs on *S. oneidensis* cell surface decreased the particle size of the extracellular biogenic AgNPs and Ag₂S NPs, suggesting that it may be possible to control particle size of the extracellular biogenic NPs via controlled expression of the genes encoding surface proteins.

3.3.3. Antibacterial Activity of AgNPs

We have shown that the particle size of the NPs from WT and the mutant lacking OMCs was different. The next question is whether the NPs from these two strains have different activities. For the biogenic AgNPs, we conducted a bacterial toxicity study using *E. coli* as a model organism to evaluate the bactericidal activity of the NPs from the WT and the OMCs mutant.

The MIC of the biogenic AgNPs from the WT and the mutant was determined to be 16 and 12 µg/mL, respectively, implying the AgNPs produced by the mutant were more toxic

to *E. coli* than those produced using the WT. To further elucidate the bactericidal activity, IC₅₀ of these biogenic AgNPs was determined. The IC₅₀ value of the AgNPs produced by WT and the OMCs mutant was estimated as 2.1 ± 0.2 and 1.2 ± 0.3 $\mu\text{g/mL}$, respectively (Figure 3.2). The IC₅₀ test was consistent with the observations in MIC assay and also suggested that the biogenic AgNPs produced using the OMCs mutant were more toxic.

Figure 3.2. IC₅₀ values (n = 8) of the biogenic AgNPs produced by WT and $\Delta(mtrC-omcA)$ (bars), and the dissolution of Ag^+ (n = 3; circles).

To investigate whether the difference in toxicity was caused by dissolution of Ag^+ from the biogenic AgNPs, we quantified Ag^+ concentration in buffers containing 100 $\mu\text{g/mL}$ AgNPs (approximately five- to eightfold higher than the MIC, ~50- to 80-fold higher than

the IC_{50}) after 24 h incubation (Figure 3.2). Concentrations of Ag^+ dissolved from the biogenic AgNPs produced by WT and the OMCs mutant were 0.40 ± 0.05 and 0.42 ± 0.06 $\mu g/mL$, respectively. There was no difference in the measured dissolution of Ag^+ from the AgNPs produced by WT and the mutant, indicating that the relative increased toxicity of the biogenic AgNPs from the mutant was not due to the dissolution of Ag^+ . Particle size could be one of the factors that may have contributed to the observed difference in toxicity. It has been reported that smaller AgNPs (especially particles <5 nm) with higher surface areas could compromise cell membrane more effectively allowing more Ag atoms to attack or enter the cells (Morones, Elechiguerra et al. 2005, Nel, Xia et al. 2006, Choi and Hu 2008). Our results on the toxicity of the biogenic AgNPs are consistent with these recent findings obtained from chemically synthesized NPs.

3.3.4 Catalytic activity of Ag_2S NPs

For the biogenic Ag_2S NPs, we used the reduction of MV^{2+} to $MV^{\cdot+}$ as a model reaction to evaluate their catalytic activity. Figure 3.3 shows MV^{2+} reduction catalysed by the Ag_2S NPs produced by WT.

Figure 3.3 Kinetic curves of the accumulation of $MV^{\bullet+}$ in solutions containing Na_2S in the absence of Ag_2S NPs, and in the presence of cell-associated or non-cell-associated Ag_2S NPs produced by *S. oneidensis* MR-1 WT.

In aqueous phase without Ag_2S NPs as the catalyst, MV^{2+} reduction by Na_2S is a very slow process. Both the Ag_2S NPs associated with cells and the Ag_2S NPs extracted from the supernatant fraction substantially accelerated the MV^{2+} reduction process. The concentration of a key reduction product $MV^{\bullet+}$ rapidly increased within the first 5–10 min and then reached an equilibrium state due to a reverse reaction between reduction products that has been described elsewhere (Kryukov, Stroyuk et al. 2004). Surprisingly, the Ag_2S NPs extracted from supernatant fraction exhibited significantly higher catalytic

activity than the Ag₂S NPs associated with the cells as indicated by an initial reduction rate of 0.26 $\mu\text{M/s}$ (non-cell-associated) and 0.09 $\mu\text{M/s}$ (cell-associated), respectively. In traditional biosynthesis of metallic NPs, the cell-associated NPs (or “metalised” cells) are harvested as potential bio-NPs catalysts, excluding non-cell-associated NPs. “Metalised” cells such as “palladized” cells have been used to catalyse a variety of reactions, including reduction of Cr(VI), ClO, trichloroethylene (TCE), and polychlorobiphenyls (PCB) as well as synthesis of organic compounds (Hennebel, De Gusseme et al. 2009, Sobjerg, Gauthier et al. 2009, Gauthier, Søjberg et al. 2010, De Corte, Hennebel et al. 2012, Hennebel, De Corte et al. 2012). Here, our results reveal that the usually neglected fraction of non-cell-associated NPs in culture supernatants showed high catalytic activity. In fact, a threefold increase in the catalytic activity could be expected by retaining the non-cell-associated NPs. Our findings highlight the need of novel methods that can potentially retain extracellular NPs effectively in the NPs biosynthesis processes. One promising strategy to achieve this could be the use of biofilms, surface-associated microbial communities encased in self-produced extracellular polymeric matrix, to immobilise in situ generated NPs via associations with both the cells and the biofilm matrix. To better understand the catalytic activity of these non-cell-associated NPs, we further investigated how their catalytic activity is influenced by the OMCs. Figure 3.4 shows that the non-cell-associated Ag₂S NPs from the mutant lacking OMCs exhibited higher catalytic activity than those from the WT. The initial reduction rate was 0.80 and 0.30 $\mu\text{M/s}$ for the Ag₂S NPs from the OMCs mutant and the WT, respectively. The results suggest that it may be possible to enhance catalytic activity of the extracellular biogenic NPs via manipulating the genes encoding surface proteins.

Figure 3.4. Kinetic curves of the accumulation of $MV^{\bullet+}$ in solutions containing Na_2S in the presence of non-cell-associated Ag_2S NPs produced by *S. oneidensis* MR-1 WT and $\Delta(mtrC-omcA)$.

3.4. Conclusion

In this study, we show that OMCs influence the particle size of extracellular NPs and their activities: (i) the lack of OMCs on *S. oneidensis* cell surface decreased the particle size of the extracellular biogenic AgNPs and Ag_2S NPs; (ii) the biogenic AgNPs from the OMCs mutant showed higher antibacterial activity than those from the WT; and (iii) the biogenic Ag_2S NPs from the OMCs mutant exhibited higher catalytic activity. Our results suggest that it may be possible to control particle size and activity of the extracellular

biogenic NPs via controlled expression of the genes encoding surface proteins. In addition, we also reveal that in extracellular biosynthesis of NPs the usually neglected non-cell-associated NPs could have high catalytic activity, highlighting the need of novel methods that can efficiently retain extracellular NPs in the biosynthesis processes.

CHAPTER 4: REDUCTIVE FORMATION OF PALLADIUM NANOPARTICLES BY SHEWANELLA ONEIDENSIS: ROLE OF OUTER MEMBRANE CYTOCHROMES AND HYDROGENASES

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4.1. Introduction

Dissimilatory metal-reducing bacteria (DMRB) are a group of microorganisms capable of using various metal(loid)s as terminal electron acceptors for anaerobic respiration (Lovley 1993, Nealson 1997). In natural environments, DMRB-mediated reduction of iron and other metal(loid)s in the Earth's crust plays an important role in biogeochemical cycles of these metals and in the fate of organic matter (Lovley 1993, Nealson 1997, Lovley and Anderson 2000). In engineered settings, there have been many attempts at harnessing the power of DMRB for various applications including bioremediation of environmental contaminants, generation of power or high value products in bioelectrochemical devices (Fredrickson, Romine et al. 2008, Nealson and Finkel 2011, Lovley 2012).

DMRB are capable of changing the oxidation state of metals and these microbial processes have opened up a new window for novel applications including biosynthesis of metal nanomaterials (Gadd 2010, Lloyd, Byrne et al. 2011). Because the synthesis of nanomaterials using DMRB usually occurs in water under gentle and environmentally

benign conditions, it has become one attractive research focus in current green nanotechnology (Mandal, Bolander et al. 2006, Hennebel, De Gusseme et al. 2009). Among the DMRB most often used in biosynthesis of nanomaterials, *Shewanella oneidensis* has attracted great interest because it is able to reduce a diverse range of metal ions and the extracellular electron transport chains responsible for transferring electrons across cell membranes have been relatively well characterized (Myers and Nealson 1988, Heidelberg, Paulsen et al. 2002, Fredrickson, Romine et al. 2008). *S. oneidensis* has been used to synthesize various nanomaterials including catalytic palladium (Pd) nanoparticles with great potentials for applications in the treatment of environmental pollutants and in chemical synthesis (De Corte, Hennebel et al. 2012, Hennebel, De Corte et al. 2012). However, the primary molecular sites responsible for Pd reduction have not been elucidated, which limits further enhancement of Pd reduction and the design of controllable and reproducible bioprocesses.

S. oneidensis MR-1 genome possesses 42 c-type cytochromes many of which are known to be involved in extracellular electron transfer (Fredrickson, Romine et al. 2008). The metal-reducing (MTR)-pathway consisting of a set of proteins including c-type cytochromes CymA and MtrC/OmcA is a well-characterized extracellular electron transport chain responsible for transferring electrons across cell membranes (Richardson, Butt et al. 2012, Shi, Rosso et al. 2012). CymA is required to electrically charge the outer membrane cytochromes (OMCs), i.e., MtrC and OmcA. Extensive studies have suggested an essential role of MtrC/OmcA in the reduction of various metals including Fe(III), Cr(VI), Tc(VII) and U(VI) (Marshall, Beliaev et al. 2006, Belchik, Kennedy et al. 2011). In addition to c-type cytochromes, hydrogenases comprise another class of redox

proteins that play a central role in microbial reduction of metals in *S. oneidensis*. The genome of *S. oneidensis* MR-1 encodes both a [FeFe]-hydrogenase HydAB and a [NiFe]-hydrogenase HyaAB (Heidelberg, Paulsen et al. 2002). HydA functions as a hydrogen-forming hydrogenase, while HyaB can function either in formation or oxidation of hydrogen as a bidirectional hydrogenase (Meshulam-Simon, Behrens et al. 2007). Previous studies have shown that the [NiFe]-hydrogenase of MR-1 was involved in H₂-driven reduction of Tc(VII) to Tc(IV)/Tc(V) (Marshall, Plymale et al. 2008, Shi, Belchik et al. 2011). However, the contribution of OMCs and hydrogenases to Pd bio-reduction in *S. oneidensis* has not been well elucidated.

The objective of the current study was to investigate the role of hydrogenases, i.e. HydA and HyaB, and OMCs, i.e., MtrC and OmcA, in bio-reductive formation of Pd nanoparticles by *S. oneidensis* MR-1. We compared the rates of Pd reduction in the wild-type (WT) and the mutants lacking OMCs and [NiFe]-hydrogenase. We demonstrated that the reduction of Pd by *S. oneidensis* can be well described by the first-order model. Our results showed that there is no significant difference in Pd reduction rates of WT and mutant lacking OMCs, while the reduction rate was significantly lower in the mutant lacking [NiFe]-hydrogenase HyaB than in the WT. Taken together, we report for the first time that outer membrane c-type cytochromes MtrC/OmcA do not play an essential role in Pd(II) reduction, while [NiFe]-hydrogenase HyaB facilitates Pd(II) reduction, suggesting that HyaB may serve as a potential target for molecular manipulation in *S. oneidensis* towards an improved performance in reductive formation of Pd nanoparticles.

4.2. Materials and Methods

4.2.1. Bacterial Strains and Growth Conditions

S. oneidensis MR-1 wild-type (WT) and mutants lacking selected c-type cytochrome or hydrogenase (Table 4.1) were used in this study. Stock cultures were maintained in LB medium with 20% glycerol at -80°C . The bacterial cells were grown in LB medium or modified M1 medium under aerobic or anaerobic conditions. The modified M1 medium (pH ~ 7.0) consisted of 3.00 mM PIPES, 7.50 mM NaOH, 26.04 mM NH_4Cl , 1.34 mM KCl, 4.35 mM NaH_2PO_4 and 0.68 mM CaCl_2 supplemented with trace amounts of minerals, vitamins, and amino acids (Cao, Shi et al. 2011). For bacterial growth in M1 medium, 20 mM sodium lactate or sodium formate was used as an electron donor and the sole carbon source. For growth under anaerobic conditions, the medium containing 10 mM ferric nitrilotriacetate (Fe(III)-NTA) as an electron acceptor was bubbled with nitrogen gas for 5 min and the headspace in the culture was less than 3% of the total volume.

Table 4.1. *S. oneidensis* strains used in this study

Strains	Description	Reference
MR-1 (WT)	Manganese-reducing strain	(Myers and Nealson 1988)
$\Delta mtrC\Delta omcA$	Deletion of SO1778 (<i>mtrC</i>)-SO1779 (<i>omcA</i>)	(Reardon, Dohnalkova et al. 2010)
$\Delta cymA$	SO4591 (<i>cymA</i>) deletion derivative of MR-1	(Marshall, Plymale et al. 2008)

$\Delta hydA$	SO3920 (<i>hydA</i>) deletion derivative of MR-1	(Meshulam-Simon, Behrens et al. 2007)
$\Delta hyaB$	SO2098 (<i>hyaB</i>) deletion derivative of MR-1	(Meshulam-Simon, Behrens et al. 2007)
$\Delta hyaB\Delta hydA$	Deletion of SO2098 (<i>hyaB</i>)-SO3920 (<i>hydA</i>)	(Marshall, Plymale et al. 2008)

4.2.2. Pd(II) reduction by resting cells

All chemicals were from Sigma Aldrich, unless otherwise stated. Anaerobic cultures of various *S. oneidensis* strains (Table 4.1) were incubated with sodium fumarate as an electron acceptor for 16 hours at 30 °C with shaking at 200 rpm. Cells were harvested through centrifugation (6000 × g for 30 min), washed twice with HEPES buffer (30 mM, pH 7.0), and resuspended in the same buffer. Ten ml of the cell suspension was mixed with 5 ml of HEPES buffer (30 mM, pH 7.0) in an anaerobic tube capped with butyl rubber stopper, and bubbled with nitrogen gas for 5 min. Sodium formate and sodium tetrachloropalladate (Na₂PdCl₄) (Pd(II)) were introduced into the anaerobic tube through syringe needles to a final concentration of 20 mM and 1 mM, respectively. Aliquots of each cell suspension were incubated at 90 °C for 10 min and were used as controls containing heat-killed cells. Each experiment was carried out in triplicates.

4.2.3. Quantification of Pd(II)

Samples (~1 ml each) were taken using a syringe needle at pre-determined time points. Each sample was centrifuged at 10 000 × g for 5 min to remove the cells and the

concentration of Pd(II) was quantified using a colorimetric method (Rotaru, Jiang et al. 2012). Briefly, 100 μ l of sample was mixed with 500 μ l of EDTA–NaOH buffer (100 mM, pH 10.4) and 50 μ l 4-(2-pyridylazo)-resorcinol (0.1%, pH 10.5) followed by 10 min incubation at 80 °C and then at 4 °C for 20 min. The mixture was then diluted 10 times using MilliQ water and the optical density at 515 nm was measured.

4.2.4. Epifluorescence microscopy

One drop of the cell suspension was placed on a glass microscope slide and dried in an oven at 70 °C. The dried sample was then stained using 4'-6-diamidino-2-phenylindole (DAPI) (Bunge, Søbberg et al. 2010). Briefly, 5 μ l of DAPI solution (1 mg ml⁻¹) was dropped onto the sample and incubated at room temperature, in the dark, for 45 min. The glass slide was then rinsed with ultrapure water to remove excess DAPI. The stained samples were examined using an inverted epifluorescence microscope (Microscope Axio Observer.Z1, Carl Zeiss).

4.2.5. Transmission electron microscopy (TEM)

For TEM imaging, samples were fixed, dehydrated and embedded in LR white (Electron Microscopy Sciences, Hatfield, PA) following protocols reported elsewhere (Belchik, Kennedy et al. 2011). Briefly, the sample was fixed in 2.5% glutaraldehyde overnight and centrifuged at 5000 $\times$ g for 5 min, and the supernatant was discarded. The cells were resuspended and washed three times with 10 mM PIPES buffer (pH 6.8) at 5000 $\times$ g for 5 min. Gradual dehydration was carried out in an ethanol series of 30, 50, 75, and 90% for 30 min each and then 100% ethanol three times for 1 h each. The cell pellet was further washed for 30 min in a 50:50 mixture of ethanol and LR white (Electron Microscopy

Sciences, Hatfield, PA). Finally, the cell pellet was washed three times using resin for 1 h each, and centrifuged at 5000 rpm for 5 min. After the final wash with resin, the resin was discarded, and the Eppendorf tube containing the cell pellet was filled with fresh resin to about 75% of the tube's volume and incubated at 60 °C overnight. The polymerized block of cell pellets was sectioned with a diamond knife (Diatome, Biehl, Switzerland) to a thickness of 70 nm and mounted on a Cu grid and sputtered with carbon. These sections were examined using a Jeol JEM-1230 TEM instrument operated at an accelerating voltage of 120 kV.

4.3. Results and Discussion

4.3.1. Pd(II) reduction by resting cells follows first-order Monod model

Upon exposure to Pd(II), cell suspension of the WT gradually turned black, suggesting that Pd(II) was reduced by *S. oneidensis* to Pd(0), which was further confirmed by the decrease in Pd(II) concentration in the aqueous phase and the formation of Pd(0) crystals in the cultures revealed by energy-dispersive X-ray spectroscopy and X-ray diffraction. Experiments conducted with various initial Pd(II) concentrations showed that the Pd(II) reduction rate by MR-1 cell suspension increased with increasing initial Pd(II) concentrations (Figure 4.1).

Figure 4.1. The rate of Pd(II) reduction by *S. oneidensis* with formate as an electron donor measured as a function of initial Pd(II) concentration at 30 °C. The solid line represents the best fit. The rate of Pd reduction can be well described by a first-order model – $d[\text{Pd(II)}]/dt = k_1 \times [\text{Pd(II)}]$ where the parameter k_1 was determined to be 164.3 h⁻¹ per cell in the reduction of Pd(II) within the concentration range of 0–4 mM.

Monod model and its derivatives under no-growth conditions have been used to describe the bioreduction kinetics for metals such as Fe(III), Co(II), U(VI), Cr(VI) and Tc(VII) (Liu, Gorby et al. 2002). Reductive formation of Pd(0) nanoparticles by *S. oneidensis* has been previously reported (Windt, Aelterman et al. 2005, De Windt, Boon et al. 2006) and the biogenic Pd(0) nanoparticles have been characterized and applied to various applications

(De Corte, Hennebel et al. 2012, Hennebel, De Corte et al. 2012). However, bioreduction kinetics for Pd(II) reduction has never been elucidated. Here, we demonstrated that Pd(II) reduction by *S. oneidensis* could be well described by a first-order model derived from the Monod model. No inhibition effects were incorporated in the first-order model, suggesting that the (by)products of the Pd(II) reduction have no significant effects on the bioreduction activity. Although the first-order model fits our macroscopic Pd(II) reduction results well, a fundamental understanding of the reduction mechanisms is required for a better interpretation of the kinetics. Bioreduction of Pd(II) by microorganisms in the presence of an electron donor is believed to occur in three concomitant steps: (i) biosorption of Pd(II) onto cell surfaces, (ii) bioreduction of Pd(II) to Pd(0), and (iii) autocatalytic reduction of Pd(II) on Pd(0) nuclei (Rotaru, Jiang et al. 2012). Thus, the macroscopic reduction results are most likely a combination of enzyme-mediated bioreduction and Pd(0)-based autocatalytic reduction. Enzymatic reduction is expected to play an important role, especially at the initial stage and under conditions with high cell-to-Pd(II) ratios. The complex biochemical and redox properties of bacterial surfaces pose a significant challenge to comprehensively uncover the underlying Pd(II) bioreduction mechanisms.

4.3.2. Outer membrane c-type cytochromes MtrC/OmcA do not play an essential role in Pd(II) reduction

The cell suspension was further examined using light microscopy by overlay of bright-field and fluorescence imaging of DAPI-stained cells as well as using TEM. Representative images are shown in Figure 4.2. In Figure 4.2A, the black precipitates with a size of hundreds nm could be the aggregates of nanoparticles. The black

precipitates were further extracted and the particle size distribution was analyzed. We found that, after ultrasonication, the size of the nanoparticles was in the range of 8–50 nm with the majority in the range of 10–20 nm in diameter. Figure 4.2B shows that the nanoparticles could be formed in the cell membrane.

Figure 4.2. (A) Dispersion of Pd(0) nanoparticles (black) in a suspension of cells (green). Cells were stained with DAPI. For enhanced clarity, the image was pseudo-colored. (B) TEM image showing the association of Pd(0) nanoparticles with *S. oneidensis* MR-1.

A significant amount of Pd(0) nanoparticles were found in the cell exterior and associated with cell membrane, suggesting an important role of extracellular and/or membrane-bound proteins in Pd(II) reduction.

Previous studies have shown that OMCs, i.e., MtrC and OmcA, play an essential role in the reduction of various metals including Fe(III), Cr(VI), Tc(VII) and U(VI) (Marshall, Beliaev et al. 2006, Belchik, Kennedy et al. 2011). To elucidate whether the OMCs, i.e., MtrC and OmcA, facilitate Pd(II) reduction, we compared Pd(II) reduction by *S. oneidensis*

MR-1 WT and the cytochrome mutants with lactate or formate as electron donors and the results are shown in Figure 4.3.

Figure 4.3. Specific rates of Pd(II) reduction (determined for the first 3 h) by *S. oneidensis* MR-1 WT and OMC mutants with 20 mM lactate or formate as electron donors in 30 mM HEPES buffer (pH 7.0) under no-growth conditions. Data represent the mean and standard deviation (n = 3).

The experimental data demonstrated that no significant Pd(II) reduction occurs in the absence of live cells, suggesting an important role of enzymatic reduction. Intriguingly, our results showed that the mutants lacking MtrC/OmcA have a comparable capability of Pd(II) reduction with that of the WT, implying that the OMCs, MtrC/OmcA, do not play an essential role in Pd(II) reduction under our tested conditions. In a recent study, Bucking

et al. reported a OMCs-independent reduction of extracellular electron acceptors in *S. oneidensis*, where two point mutations in MtrA and MtrB were found to regain the ability of the mutant lacking OMCs to grow as a dissimilatory metal reducer (Bücking, Piepenbrock et al. 2012). Hence, we further tested the mutant lacking CymA, a cytochrome transferring electrons to MtrA, in Pd(II) reduction. Interestingly, the mutant $\Delta cymA$ reduced Pd(II) as fast as the WT, suggesting that the MTR-pathway does not play an essential role in Pd(II) reduction by *S. oneidensis*. As one well-characterized extracellular electron transport chain in *S. oneidensis*, the MTR-pathway getting electrons from the quinone pool through CymA has been reported to be responsible for transferring electrons across cell membranes to a wide variety of extracellular electron acceptors, especially insoluble electron acceptors such as ferrihydrite (Shi, Squier et al. 2007, Shi, Rosso et al. 2012). Here we show that the MTR-pathway is not essential for Pd(II) reduction, implying that the electrons for Pd(II) reduction are taken from the upstream of the electron carrier CymA, possibly at the quinone pool level.

4.3.3. [NiFe]-hydrogenase HyaB facilitates Pd(II) reduction

In addition to OMCs, hydrogenases comprise another class of redox proteins that can take electrons from the quinone pool and they have also been shown to be involved in the reduction of Tc(VII) to Tc(IV)/Tc(V) (Marshall, Plymale et al. 2008, Belchik, Kennedy et al. 2011). The redox potential of the [Pd(II)Cl₄]²⁻/Pd(0)(s) couple is 600 mV at the standard state (Atkins 2010). The [4Fe–4S] clusters of the [NiFe]-hydrogenase have mid-point potentials of around –300 mV (Teixeira, Moura et al. 1989), indicating that it is thermodynamically feasible for hydrogenases to reduce Pd(II) to Pd(0).

To test the involvement of hydrogenases in Pd(II) reduction in *S. oneidensis*, the capability of the mutants lacking hydrogenases in reducing Pd(II) in the presence of formate as electron donors was compared with that of the WT (Figure 4.4).

Figure 4.4. Specific rates of Pd(II) reduction by *S. oneidensis* MR-1 WT and hydrogenase mutants with 20 mM formate as electron donors in 30 mM HEPES buffer (pH 7.0) under no-growth conditions. Specific reduction rate was determined for the first 3 h of Pd(II) reduction.

The [FeFe]-hydrogenase mutant (ΔhydA) exhibited a comparable reduction rate to the WT, while both deletions of the [NiFe]-hydrogenase (ΔhyaB and $\Delta\text{hyaB}\Delta\text{hydA}$) have significantly lower reduction rates than the WT. The results suggested that the [NiFe]-hydrogenase HyaB is a key enzyme facilitating Pd(II) reduction by *S. oneidensis*.

The involvement of unspecified hydrogenases in Pd(II) reduction by *Desulfovibrio desulphuricans* has been implicated by the inhibition of the bioreduction with 0.5 mM Cu²⁺, a hydrogenase inhibitor (Lloyd, Yong et al. 1998). However, the presence of Cu²⁺ could lead to conformational changes in proteins in general (Vardanyan and Trchounian 2010); hence, the inhibition might not be specific for hydrogenases. The direct evidence supporting the involvement of hydrogenases in bioreduction of Pd(II) by *D. fructosivorans* and *E. coli* was reported using mutant strains lacking hydrogenases (Mikheenko, Rousset et al. 2008, Deplanche, Caldelari et al. 2010). Here in this study, we showed that the periplasmic [NiFe]-hydrogenase HyaB played an important role in mediating Pd(II) reduction. It should be noted that, although [NiFe]-hydrogenase HyaB plays an important role in facilitating Pd(II) reduction, other enzymes may also mediate Pd(II) reduction as suggested by ~50% of reduction capability remaining in the mutant strain lacking HyaB (Figure 4.4).

4.3.4. Proposed mechanisms of hydrogenase-facilitated Pd(II) reduction

Based on our results, we proposed a working model for the mechanisms of hydrogenase-facilitated Pd(II) reduction in *S. oneidensis* (Figure 4.5).

Figure 4.5. Model of hydrogenase-facilitated Pd(II) reduction by *S. oneidensis* with formate as electron donors.

The hydrogenases of *S. oneidensis* are located in the periplasm (Meshulam-Simon, Behrens et al. 2007, Marshall, Plymale et al. 2008, Shi, Belchik et al. 2011). The [FeFe]-hydrogenase HydAB and a putative formate dehydrogenase FDH form a formate-hydrogen lyase (FHL) complex, mediating the conversion of formate to hydrogen, while the [NiFe]-hydrogenase HyaB can function bidirectionally in both formation and oxidation of hydrogen (Meshulam-Simon, Behrens et al. 2007). Using formate as electron donors, the cells may produce hydrogen via the FHL activity as well as the FDHs and HyaB activity. Hydrogen can be oxidized by HyaB coupling to the reduction of Pd(II). The reduced Pd products, presumably Pd(0) nanoparticles, are most likely accumulated in the periplasm.

The released protons are then transported into the cytoplasm by proton transporters such as ATP synthase located in the inner membrane.

4.4. Conclusion

The reduction of Pd(II) by *S. oneidensis* can be well described by a first-order model. The OMCs, i.e., MtrC/OmcA, do not play an essential role in Pd(II) reduction under our tested conditions, while [NiFe]-hydrogenase (HyaB) facilitates Pd(II) reduction using lactate or formate as electron donors. Our findings suggest that [NiFe]-hydrogenase HyaB may serve as a potential target for molecular manipulation in *S. oneidensis* towards an improved performance in reductive formation of Pd nanoparticles.

CHAPTER 5: ENGINEERING BIOFILM WITH NANOCATALYTIC ACTIVITY THROUGH *IN SITU* BIOREDUCTIVE FORMATION OF PALLADIUM NANOPARTICLES IN THE BIOFILM MATRIX

A portion of this chapter has been presented in oral format as Ng CK and Cao B (2016). Engineering biofilm for remediation of environmental Chromium(VI) contamination. 6th International Conference on Environment Science and Biotechnology, Kyoto, Japan and in poster format as Ng CK, Ding Y and Cao B (2014). Understanding and harnessing the power of biofilm matrix: What we have learned from Shewanella biofilms. Biofilm6, Vienna, Austria.

5.1. Introduction

Biocatalysis using enzymes or microbial cells is an environmentally friendly process that has been increasingly applied to biotransformation in chemical industry (Bornscheuer, Huisman et al. 2012). Application of whole cells as biocatalysts is often preferred over isolated enzymes primarily because of higher catalyst stability, readily available self-generated cofactors, and relatively lower cost for whole cell biocatalysts (Woodley 2006). However, although whole cell biocatalysis has been extensively studied, it is so far mainly limited to batch and fed-batch processes where planktonic cells are used as the workhorse (Rosche, Li et al. 2009). The performance of biocatalysis is often hampered by chemical toxicity imposed by substrate and/or product (Gross, Hauer et al. 2007). In addition, cell retention and long-term viability are among the challenges that are posed to more cost effective continuous bioprocesses (Rosche, Li et al. 2009).

Microbial biofilms might deliver a solution. Biofilms are structured, surface-associated, microbial communities that are prevalent in most natural environments, engineering

systems and clinical settings. One of the most important features of a biofilm is the presence of extracellular polymeric substances (EPS) that form a matrix and encase the cells in the biofilm. Extensive studies have demonstrated a wide range of advantages of the presence of EPS matrix for the biofilm mode of life (Flemming and Wingender 2010, Flemming 2011). Thanks to the EPS matrix, cells in biofilms often form structurally stable communities and show a remarkable resistance against various biocides. In biofilm-based biocatalysis, EPS matrix may offer the biocatalysts the advantages of self-immobilisation and long-term activity. In addition, the robust structures of biofilms might render them capable of tolerating harsh physicochemical conditions such as toxic reactants or solvents, allowing continuous biocatalysis for reactions that are biologically challenging for planktonic cells (Rosche, Li et al. 2009, Halan, Schmid et al. 2010). To date, the use of biofilms in biocatalysis processes has been largely confined to wastewater treatment (Cohen 2001, Wuertz, Bishop et al. 2003, Singh, Paul et al. 2006). The exploration of applying biofilms as biocatalysts in chemical industry is still in its infancy due to the fact that the analyses and control of biofilms-based bioprocesses are very challenging because of the intrinsic highly heterogeneous and spatiotemporally dynamic properties of biofilms (Rosche, Li et al. 2009, Halan, Schmid et al. 2010). With an enhanced understanding of biofilm biology, the use of biofilms as biocatalysts for the synthesis of simple organic compounds such as converting benzaldehyde to benzyl alcohol and styrene to (*S*)-styrene oxide has been reported (Li, Webb et al. 2006, Gross, Hauer et al. 2007, Halan, Schmid et al. 2010). To further engineer the catalytic activities of the biofilms, recombinant bacterial strains capable of producing certain enzymes for specific biotransformation have been explored (Tsoligkas, Winn et al. 2011, Winn, Foulkes et al.

2012). For industrial applications, multifunctional catalysts are usually required. To this end, constructing biofilms containing multiple species catalysing a series of reactions in a type of reaction cascade has been suggested as an interesting approach (Stubblefield, Howery et al. 2010). However, the development of robust, functionally reproducible and controllable multispecies biofilms is hampered by limited understanding of dynamic interspecies interactions in biofilms.

The ability of certain microorganisms to reduce metal ions to zero-valent metallic nanoparticles may provide a novel strategy for developing multifunctional biocatalysts. Precious metals such as palladium (Pd) have been widely used as catalysts for numerous reactions in chemical, pharmaceutical, automotive and electronics industries (Das 2010). Microorganisms have the potential to reduce soluble Pd species to zero-valent Pd, i.e., Pd(0), resulting in the formation of highly reactive Pd(0) nanoparticles that may be directly used in various nanocatalysis conversions (Hennebel, De Gusseme et al. 2009, Gadd 2010, Lloyd, Byrne et al. 2011, De Corte, Hennebel et al. 2012, Hennebel, De Corte et al. 2012). Hence, we hypothesize that it may be possible to engineer biofilms with nanocatalytic activities through bioreduction of Pd and biofilm matrix-facilitated immobilisation of the reduction products, i.e., Pd(0) nanoparticles.

To test this hypothesis, we used *Shewanella oneidensis* as a model organism because it is a well-documented metal-reducing bacterium capable of reducing a wide range of metal ions including Pd and the extracellular electron transport chains responsible for moving electrons across cell membrane have been relatively well characterized (Shi, Squier et al. 2007, Fredrickson, Romine et al. 2008, Shi, Richardson et al. 2009, Richardson, Edwards et al. 2012, Shi, Rosso et al. 2012). The objectives of this study were (i) to explore the

reductive formation of Pd(0) nanoparticles in biofilms (or “nanocatalysation”) under ambient conditions, (ii) to understand the metabolic responses of the biofilms during the “nanocatalysation” process, (iii) to develop a robust nanocatalytic biofilm through genetic engineering. This study demonstrated for the first time the engineering of biofilms for nanocatalytic activity through in situ formation of nanocatalysts in biofilm matrix and influencing the robustness of the biofilm via increasing intracellular cyclic-di-GMP level. This study provided the basis for a novel strategy to develop robust, multifunctional biofilm-based biocatalysts.

5.2. Materials and Methods

5.2.1. Bacterial strains and growth conditions

E. coli and *S. oneidensis* MR-1 wild-type (WT) and mutants used in this study are described in Table 5.1. Stock cultures were maintained in LB medium with 20% glycerol at -80°C. The bacterial cells were grown in LB medium or modified M1 medium under aerobic or anaerobic conditions. The modified M1 medium (pH ~7.0) consisted of 3.00 mM PIPES, 7.50 mM NaOH, 26.04 mM NH₄Cl, 1.34 mM KCl, 4.35 mM NaH₂PO₄ and 0.68 mM CaCl₂ supplemented with trace amounts of minerals, vitamins, and amino acids (Cao, Shi et al. 2011). For bacterial growth in M1 medium, 20 mM sodium lactate or sodium formate was used as an electron donor and the sole carbon source. For growth under anaerobic conditions, the medium containing 10 mM ferric nitrilotriacetate (Fe(III)-NTA) as an electron acceptor was bubbled with nitrogen gas for 5 min and the headspace in the culture was less than 3% of the total volume.

Table 5.1. *S. oneidensis* strains used in this study.

Strains / Plasmid	Description	References
Strains		
<i>S. oneidensis</i> MR-1	Manganese-reducing strain	(Myers and Nealson 1988)
$\Delta mtrC$	Deletion derivative of SO1778 (<i>mtrC</i>) within MR-1	(Marshall, Plymale et al. 2008)
$\Delta omcA$	Deletion derivative of SO1779 (<i>omcA</i>) within MR-1	(Marshall, Plymale et al. 2008)
<i>E. coli</i> DH5α	Laboratory strain	(Zhang, Ng et al. 2014)
Plasmids		
<i>pYedQ2</i>	Gm ^r ; pBBR1MCS3 carrying the YedQ gene	(Gjermansen, Ragas et al. 2006)
<i>pRK600</i>	Cm ^r ; oriColE1 RK2-Mob ⁺ RK2- Tra ⁺ ; helper vector for conjugation	(Kessler, Lorenzo et al. 1992)

5.2.2. Construction of c-di-GMP mutants

YedQ (previously known as *yhck*) gene from *E. coli* is cloned from the *pYedQ* plasmid in HindIII/BamHI side of pBBR1MCS-5 plasmid vector to make the *pYedQ2* plasmid.

The *S. oneidensis* MR-1 mutant that overexpresses c-di-GMP was constructed via plate mating as follows: *S. oneidensis* MR-1, *Escherichia coli* containing *RK600*, *Escherichia coli*-DH5 α - *pYedQ2* were grown using LB media overnight in shaking incubator (200 rpm) at 30 °C, 37 °C and 37 °C respectively. Antibiotics such as chloramphenicol and gentamicin were used in cell culturing when necessary. 1 ml of the culture from each of the three strains was washed two times with fresh LB medium, using centrifugation at 10000 x g for 3 minutes. The cell pellets from each of the three strains was resuspended and mixed at 1:1:1 ratio in 1 ml of LB media. Centrifugation was done to obtain a cell pellet containing cells from all three strains in equal proportions. The cell pellet was transferred to a LB agar plate and incubated in 30 °C for 5 hours. The cells were harvested from the plate via washing with 1 ml of 0.9% sodium chloride solution (saline). 100 μ l of cells were spread on a LB agar plate containing 20 μ g/ml tellurite and 60 μ g/ml gentamicin, and kept in incubator at 30 °C until transposon mutant colonies appeared. Gel electrophoresis was run with the plasmid extracted from the selected mutant as a confirmation that the selected mutant indeed contains the *pYedQ2* plasmid. Swim plate experiments were done in LB agar plates. All swim plates experiments were conducted with five replicates.

5.2.3. Growth of *S. oneidensis* biofilms and Pd(II) reduction

Three independent biofilms of *S. oneidensis* MR-1 WT and c-di-GMP mutants were grown in tubular biofilm reactors (~25 cm of 16" silicon tubings) using 10 times diluted LB medium containing 10 mM lactate continuously supplied through a peristaltic pump at a flow rate of 8 ml/h. The flow system was assembled and prepared as described previously (Sternberg and Tolker-Nielsen 2006). Each tubular reactor was inoculated by injecting 2 ml overnight culture (diluted to an OD₆₀₀ of 0.01) using a syringe. After inoculation, the medium flow was stopped for 1 h to allow initial attachment followed by continuous media flow with a flow rate of 8 ml/h.

The *S. oneidensis* biofilms were allowed to grow for 72 h, and then the feed medium was loaded with 1 mM Pd(II). Effluent samples were collected and the concentration of Pd(II) was determined using the colorimetric method described above. The same setup with no biofilms growing in it served as a control. The amount of Pd accumulated in the biofilms was estimated by subtracting the total amount of Pd(II) introduced into the biofilm reactors by the amount of Pd(II) remained in the effluents. Samples from the media effluents were taken periodically and cell detachment was evaluated by counting colony forming unit (CFU) using the drop-plate method described elsewhere (Chen, Nace et al. 2003).

Dissolved oxygen (DO) concentration was continuously monitored (5 s interval) using a flow-through optical oxygen microsensor (Pyro-Science OXFTC, Aachen, Germany) connected to the outlet of the tubular biofilm reactor. The signal from the microsensor was processed by the sensor meter (Pyro-Science FireSting O2-4, Aachen, Germany). Before starting measurements, two-point calibration was carried out in anoxic water and air-

saturated water. DO concentration at different depth in the biofilms was measured using a retractable needle-type oxygen microsensor (tip diameter ca. 50 μm) (Pyro-Science OXR50, Aachen, Germany) connected to a micromanipulator (Pyro-Science MUX2, Aachen, Germany).

5.2.4. Biofilm viscosity

The viscosity of biofilm samples was measured using a Physica MCR 301 rheometer (Anton Paar). Biofilms of WT and c-di-GMP mutant were prepared by dropping 100 μl of overnight cultures ($\text{OD}_{600} \sim 1.5$) onto 0.45 μm filter membranes (diameter 87 mm, Schleicher & Schuell, Dassel, Germany) placing on LB agar and incubating at 30°C for 24 h. The membranes with biofilms were then transferred to the rheometer and the viscosity of each biofilm was determined as previously described (Lieleg, Caldara et al. 2011, Powell, Sowedan et al. 2013).

5.2.5. Epifluorescence microscopy

After continuously exposing to Pd(II) for 2 days, the biofilm-Pd association was harvested and a portion of the biofilms were resuspended in HEPES buffer (30 mM, pH 7.0) to a final cell density of $\sim 10^9$ cells/ml. One drop of the suspension was placed on a piece of microscopy glass slide and was dried in an oven at 70°C. The dried sample was then stained using 4'-6-Diamidino-2-phenylindole (DAPI). Briefly, 5 μl of DAPI solution (1 mg/mL) was dropped onto the sample and incubated at room temperature in dark for 45 min. The glass slide was then rinsed with ultrapure water to remove excess DAPI. Then the stained samples were examined under an inverted epifluorescence microscope (Microscope Axio Observer.Z1, Carl Zeiss).

5.2.6. Field emission scanning electron microscopy and energy-dispersive X-ray spectroscopy

Biofilm samples were re-suspended in MilliQ water. A portion of each suspension was dried under vacuum and then coated with platinum using a vacuum electric sputter coater (JEOL JFC-1300, JEOL Asia Pte. Ltd., Singapore). SEM images were then taken using a Field Emission Scanning Electron Microscope (FE-SEM, JSM-7600, JEOL Asia Pte. Ltd., Singapore) at a voltage of 2.0-5.0 kV. EDX spectrum was obtained using an energy-dispersive X-ray spectroscope (AZtecEnergy, Oxford Instruments, Oxfordshire, UK).

5.2.7. X-ray diffraction

To further characterize the Pd associated with the biofilms, X-ray diffraction (XRD) analysis of the dried biofilms-Pd association was performed using a Bragg-Brentano diffractometer (Bruker D8 Advanced) in θ - 2θ configuration with a Cu K α line at 0.15418 nm as a source generated by a copper X-ray tube with power 2.2 kW (40 kV, 40 mA). Measurements were performed in the range of 2θ = 20-90 degrees.

5.2.8. Biofilm-associated Pd nanoparticles

The biofilm-Pd association was resuspended in HEPES buffer (30 mM, pH 7.0) and the cell suspensions were centrifuged at 10,000 x g for 5 min. The pellet contained cells and cell-associated Pd nanoparticles. Matrix-associated Pd nanoparticles in the supernatants were further harvested by using ultracentrifugation at 100,000 x g for 30 min, washed three times with MilliQ water and dried at 50 °C under vacuum. Both the cell- and matrix-associated nanoparticles were subjected to further analyses.

5.2.9. Size distribution and zeta-potential measurements

The hydrodynamic diameters and the zeta potential of the matrix-associated Pd nanoparticles were determined using a Zetasizer (Nano ZS, Malvern Instruments; Worcestershire, UK). For the size distribution measurement, the nanoparticles were first suspended in ultrapure water and subjected to ultrasonication for 10 min before the sample was transferred into a cuvette. Three readings were taken for each sample, and each reading was made up of the average of 12 to 21 individual measurements. For the zeta-potential measurement, each reading was made up of the average of 3 individual measurements.

5.2.10. Transmission electron microscopy

For the matrix-associated Pd nanoparticles, about 3 μ l was applied to a carbon-coated copper grid and dried in the air for transmission electron microscope (TEM) imaging using a Jeol JEM-1230 TEM instrument operated at an accelerating voltage of 120 kV. For biofilms, samples were fixed in 2.5% glutaraldehyde overnight and centrifuged at 5000 x g for 5 min, and the supernatant was discarded. The cells were resuspended and washed with 10 mM PIPES buffer (pH 6.8) at 5000 x g for 5 min (repeated three times). Gradual dehydration was carried out in ethanol series 30, 50, 75, 90% for 30 min each and then 100% ethanol 3 times for 1 h each. The cell pellet was further washed for 30 min in ethanol and LR white (Electron Microscopy Sciences, Hatfield, PA) (50:50). Finally, the cell pellet was washed 3 times using resin for 1 h each (at 5,000 x g for 5 min). After the final wash with resin, the resin was discarded, and the Eppendorf tube containing the cell pellet was filled with fresh resin (~3/4 volume) and incubated at 60°C overnight. The polymerized

block of cell pellets was sectioned with a diamond knife (Diatome, Biehl, Switzerland) to a thickness of 70 nm and mounted on a Cu grid and sputtered with carbon. These sections were examined using a Jeol JEM-1230 TEM instrument operated at an accelerating voltage of 120 kV.

5.2.11. Catalytic activity of the biofilms

The harvested biofilms were suspended in HEPES buffer (30 mM, pH 7.0), centrifuged at 6,000 x g for 20 min, and washed with the same buffer. Both the cell- and matrix-associated fractions of the nanoparticles were evaluated for their catalytic activities in dechlorination reaction. Each fraction (~10 mg, 2 mg and 12 mg for cell-nanoparticles, matrix-nanoparticles and biofilm-nanoparticles associations, respectively) was added into an anaerobic bottle containing 20 μ M sodium formate and 40 mM trichloroethylene (TCE) in HEPES buffer (30 mM, pH 7.0). The reaction system was flushed with nitrogen gas before incubating at room temperature on a shaker (200 rpm). An air-tight syringe was used to draw an aliquot of headspace from the anaerobic glass tubes at different time points. The concentration of TCE was determined using a gas chromatography as previously described (Hennebel, Simoen et al. 2009).

5.2.12. Proteomics analysis

5.2.12.1. Preparation of cellular proteins

LB cultures of *S. oneidensis* at the mid-exponential growth phase were exposed to 1, 5, and 10 mM Pd(II). After 30-min exposure, each culture was pelleted by centrifugation at 5,000 x g for 20 min, and the pellet was then washed twice using PBS buffer. One set of

cultures with no Pd(II) exposure was used as control (0 mM). The harvested cells were lysed at 4 °C in lysis buffer (0.1% SDS, 0.5 M TEAB, 50 mM protease inhibitor tablet) with intermittent vortexing and sonication. The lysate was centrifuged at 20,000 x g for 1 h at 4 °C and supernatant will be stored at -80 °C. Protein concentration was estimated using BCA assay.

5.2.12.2. iTRAQ labelling

Proteins (25 µg) of each sample was reduced with 53 mM TCEP (tris(2-carboxyethyl) phosphine) for 30 min at 37°C and alkylated using 10 mM iodoacetamide for 30 min at room temperature in the dark. After reduction and alkylation, each sample was then digested with sequencing-grade porcine modified trypsin (Promega, Madison, WI, USA) at a trypsin to protein ratio of 1:10 at 37°C for 16 h. The iTRAQ 4-plex kit (AB SCIEX, Foster City, CA, USA) was used to label the 4 samples according to the manufacturer's instructions. Protein samples from the cultures exposed to 0, 1, 5 and 10 mM Pd(II) were labelled with iTRAQ reagents 114, 115, 116 and 117, respectively. After 1 h incubation at room temperature, the 4 labelled samples were pooled into one set. To remove any unbound labels, excess trypsin and SDS, the sample was cleaned using strong cation exchange (SCX) chromatography followed by reverse phase (RP) chromatography using an Opti-Lynx cartridge holder (Applied Biosystems, Foster City, CA, USA) and a syringe pump (KD Scientific, Holliston, MA, USA) at a flow rate of 9.5 ml/h. The eluent was vacuum-dried and the peptides were re-dissolved in 0.05% heptafluorobutyric acid (HFBA)/1% formic acid.

5.2.12.3. LC-MS/MS and data analysis

Solubilised peptides were chromatographed twice (2 separate injections to provide technical replicates) by online strong cation exchange (SCX) and nano-C18 LC using an Ultimate HPLC, Switchos and Famos autosampler system (LC-Packings, Amsterdam, Netherlands). Peptide samples were loaded onto a small SCX micro trap (500 μm ×12 mm, Poros S10, AB SCIEX, Foster City, CA, USA) and were eluted sequentially with 5, 10, 15, 20, 25, 30, 40, 50, 100, 250, 500, and 1,000 mM ammonium acetate (20 μl). The initial unbound fraction and each salt step fraction were concentrated and desalted onto a micro-C18 pre-column (500 μm ×2 mm; Michrom Bioresources, Auburn, CA, USA) at 15 $\mu\text{l}/\text{min}$. After a 10-min wash, the pre-column was switched (Valco 10 port valve, Dionex) in line with a fritless nano analytical column (75 μm ×10 cm) containing C18 reverse phase media (5 μm , 200 Å, Magic, Michrom Bioresources, Auburn, CA, USA) eluted with a linear gradient of acetonitrile (ACN) in 0.1% (v/v) formic acid from 2% to 36% ACN for 74 min, followed by 1 min at 90% ACN at ~300 nl/min. The column was connected via a fused silica capillary to a low-volume tee (Upchurch Scientific, Oak Harbor, WA, USA) where high voltage (2,300 V) was applied and the column tip was positioned ~1 cm from the orifice of a QStar Elite mass spectrometer (AB SCIEX, Foster City, CA, USA). The QStar was operated in information-dependent acquisition mode. A time-of-flight (TOF) MS survey scan was acquired (m/z 350-1750). The 3 most abundant multiply charged ions (counts > 25) were sequentially selected by the quadrupole for ToF MS/MS analysis. Tandem mass spectra were accumulated for up to 2.5 s (m/z 65-2,000) using the Advanced IDA parameters automatic MS/MS accumulation (set to 20) and automatic collision energy. Protein identification and relative abundance of proteins were

determined using the ParagonTM algorithm in ProteinPilotTM 4.0 software (AB SCIEX, Foster City, CA, USA). Each MS/MS spectrum was compared with known sequences in the NCBI non-redundant. Search parameters were set to: sample type, iTRAQ 4-plex (peptide labeled); cysteine alkylation, iodoacetamide; digestion, trypsin; identification focus, biological modifications; search effort, thorough ID. Ratios of the areas under the peaks at 114, 115, 116 and 117 Da were calculated to determine a protein's relative abundance across all samples in the iTRAQ set. The following criteria were imposed on the data to identify significantly differentially abundant proteins: an unused protein score > 1.3, which corresponds to a confidence limit of 95%; at least 2 unique peptides with confidence > 95% detected; $p < 0.05$ (Student's t-test, assuming equal variance) and relative abundance changes greater than 2-fold. To correct for small differences in protein loading, the generated ratios of all detected proteins were normalised by bias correction with the ProteinPilotTM software.

5.2.13. Static Biofilm assay and Motility assay

Overnight cultures (1 uL) of *S. oneidensis* MR-1 WT and c-di-GMP mutant were inoculated into 96-well plate containing 200 uL of Lysogeny Broth (LB) medium. 40 replicates of WT, 40 replicates of c-di-GMP mutant and 16 replicates of abiotic control were setup in the 96-well plate. The cultures were allowed to grow for 12 hours and 18 hours respectively before static biofilm assay was done for the two setups, in accordance to the microtiter plate biofilm assay protocol (Merritt, Kadouri et al. 2005).

In short, the 96-well plates were briefly washed with water to remove planktonic cells. 1% crystal violet were added to stain the biofilm attached to the walls of the well for 10 minutes.

Excess crystal violet was washed away using water, and the crystal violet-stained biofilms were solubilised in 70% ethanol (200 μ L). The resultant mixture was transferred into corresponding wells in a separate, clean 96-well plate to be analysed by a microtiter plate reader for optical density at wavelength of 550 nm (OD_{550}). Using similar 96-well plate setup, WT and mutant cultures were allowed to incubate in 30 °C under no-shaking condition, and OD_{600} readings were taken every 5 minutes for 20 hours.

5.2.14. C-di-GMP extraction and quantification

Three replicates of *S. oneidensis* MR-1 WT and mutant were grown for a specific duration according to the condition of the experiment. An aliquot (2 ml) was taken from each culture and centrifuged for three minutes. The supernatant was discarded and the cell pellets were washed with 1mM ice-cold ammonium acetate. The respective cell suspensions were homogenized by passing five-times through a 20-gauge needle. After centrifugation, the cell pellets were suspended in 2-3 pellet volume of ice cold solution comprising of acetonitrile, methanol and water at 2:2:1 ratio. The resultant cell suspensions undergo ultrasound probe treatment at high power for 5 minutes in ice filled bath. The resultant cell suspensions were centrifuged and the supernatants were retained. The volume of the supernatants was reduced initially by temperature controlled speedvac to around 1 mL, while the remaining solutions were subjected to lyophilisation. The samples were then suspended in 100-200 μ L of 1mM ammonium acetate, sonicated for 10 minutes in ice cold bath, and centrifuged for 5 minutes at 10000 rpm. The supernatants were transferred to glass inserts for analysis using Liquid Chromatography as previously described (Kuchma, Ballok et al. 2010).

5.2.15. Iron reduction and quantification

Overnight cultures of *S. oneidensis* MR-1 WT and c-di-GMP mutant were subjected to centrifugation (10000 x g for 10 minutes), and the cell pellets were harvested and washed thrice using 30 mM HEPES buffer. The cells were suspended in the HEPES buffer, and the final OD₆₀₀ of 0.1 were achieved for all the cell suspensions. The cell suspensions were placed in anaerobic glass tubes capped with butyl rubber, with three replicates each for WT and c-di-GMP mutant, and each setup was bubbled with N₂ gas for 10 minutes. Sodium lactate and Fe-NTA were subsequently added into the set-up in an anaerobic chamber (Shel Lab, Cornelius, USA), to a final concentration of 20 mM and 10 mM respectively. Iron quantification was done using Ferrozine Assay as previously described (Riemer, Hoepken et al. 2004).

5.2.16. Western blotting

Culture of *S. oneidensis* MR-1 WT and c-di-GMP mutant were grown in LB media (10 ml) for 8 hours on a temperature-controlled shaking incubator (30°C, 200 rpm). The cells were harvested and washed thrice using PBS buffer. The cell pellet was suspended in 200 µl of SDS-PAGE loading buffer, boiled for 10 min at 70°C, cooled to room temperature, and incubated with 2 µl of DNase1 for 10–15 min at 37°C. Samples were then directly subjected to NuPAGE (8% Tris-Acetate gels) followed by dry protein transfer onto a nitrocellulose membrane using the iBlot system (InVitrogen). Membranes were developed using a standard Western blot protocol [primary antibody: Rabbit IgG (H+L), dilution 1:3,000; secondary antibody: anti-rabbit horseradish peroxidase, dilution

1:16,000]. Membranes were imaged using a Fuji LAS-1000 system and Image reader for LAS-1000 Pro (Fujifilm, Tokyo, Japan).

5.2.17. Biofilm extraction and quantification

Three replicates of tubing biofilm reactors for WT and c-di-GMP mutant each were set up using silicon tubing (Masterflex L/S 16, i.d. 3.2 mm) for biofilms to attach and grow in the interior wall. The lengths of all the tubing were kept constant at 20 cm, and the weights of each tubing were noted. Each tubing reactor was inoculated using diluted overnight cultures in LB medium ($OD_{600} \sim 0.15$) followed by 2-h stop-flow to allow cells to attach onto the walls of the tubing. 1/10 LB medium was then continuously supplied with a flow rate of 4 ml/h for biofilm growth. After 120 h, biofilms formed in the tubing biofilm reactors were evaluated. Biomass from the tubing were dried and weighed together with the tubing to obtain the dry mass of the biomass.

5.2.18. RNA extraction, sequencing and analysis

S. oneidensis MR-1 WT and c-di-GMP mutant cultures are inoculated in LB medium, with three biological replicates each, and incubated aerobically for 8 hours at 30°C with shaking at 200 rpm. This was followed by the isolation of RNA from MR-1 WT and mutant, using RNeasy mini Kit (Qiagen, Germany), with the DNA being depleted using RNase-free DNase set (Qiagen, Germany). All isolation procedures were conducted according to the manufacturer's instructions. No depletion of ribosomal RNA was done for this experiment. The concentration of RNA in each samples were determined using Nanodrop 2000 spectrophotometer (Thermo Scientific, USA) and were further confirmed using Qubit

2.0 Fluorometer (Life Technologies, Germany). All RNA samples were promptly stored at -80°C until further use.

3 biological replicates per strain were used in this experiment. 3 analytical replicates were further generated for each biological replicates to be sequenced. A total of 18 RNA samples from MR-1 WT and c-di-GMP mutant are sequenced by the Singapore Centre on Environmental Life Sciences Engineering (SCELSE). Quality of the RNA samples was determined using the Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen) and Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen) on a Bioanalyzer RNA 6000 Nano Chip (Agilent). Next-generation sequencing library preparation was performed by following TruSeq RNA Sample Preparation v2 protocol (Illumina) with modifications: The mRNA purification step was omitted and instead, 200 ng of total RNA was directly added to the elute-fragment-prime step. The PCR amplification step, which selectively enriches for library fragments that have adapters ligated on both ends, was performed according to the manufacturer's recommendation but the number of amplification cycles was reduced to 12. Each library was uniquely tagged with one of Illumina's TruSeq LT RNA barcodes to allow library pooling for sequencing. Library quantitation was performed using Invitrogen's Picogreen assay and the average library size was determined by running the libraries on a Bioanalyzer DNA 1000 chip (Agilent). Library concentration was normalised to 2 nM and the concentration was validated by qPCR on a ViiA-7 real-time thermocycler (Applied Biosystems), using qPCR primers recommended in Illumina's qPCR protocol and Illumina's PhiX control library as a standard. Libraries were then pooled at equal volumes and sequenced in two lanes of an Illumina HiSeq2500 rapid run at a final concentration of 7.2 pM and a read-length of 101 bp paired-end.

The Illumina reads were functionally assigned by mapping against the published genome of *S. oneidensis* MR-1 using CLC Genomics Workbench (CLC bio, Aarhus, Denmark). CLC Genomics workbench is a commercially-available program which is able to calculate gene expression levels, using an approach previously described (Mortazavi, Williams et al. 2008). The mRNA reads were normalised using the RPKM (reads assigned per kilobase of target per million mapped read) method. A t-test using RPKM normalised values with FDR (False Discovery Rate) p-value correction were performed and genes with p-value equal or more than 0.01 are rejected. Genes with the n-fold changes were computed using the RPKM values, converted into Log2-scale, and only those genes with 2-fold Log2-scale changes in expression were considered significant.

5.3. Results and discussion

5.3.1. Biofilms reduce Pd(II) and accumulate Pd(0) nanoparticles under aerobic conditions

It has been shown that hydrogenase HyaB but not the outer membrane c-type cytochromes MtrC/OmcA plays significant roles in mediating Pd(II) reduction (Ng, Cai Tan et al. 2013). MtrC and OmcA can be expressed under both aerobic and anaerobic conditions, while HyaB is expressed only under anaerobic conditions. Further, under aerobic conditions, oxygen competes strongly with Pd(II) for electrons. Thus, one would expect that creating anaerobic conditions may enhance Pd(II) reduction. Highly heterogeneous microenvironments in biofilms might provide favourable conditions for Pd(II) reduction even under aerobic conditions. To test the idea of engineering biofilms via Pd(II) reduction under aerobic conditions, we grew *S. oneidensis* MR-1 biofilms in

tubular reactors and then exposed the biofilms to Pd(II). Anoxic zones with low or no dissolved oxygen were observed in the biofilms grown under aerobic conditions (Figure 5.1), which supports our hypothesis that favourable conditions for Pd(II) reduction may develop in biofilms even under aerobic conditions.

Figure 5.1. A) Graph showing dissolved oxygen (DO) concentration from various distance from the bottom of the biofilm. B) Schematic diagram showing the reduction of Pd(II) ions to Pd(0) nanoparticles within the biofilm under bulk oxic condition.

The biofilms were grown in tubular biofilm reactors for 3 days and the 3-day old biofilms were then exposed to 1 mM Pd(II) ($t = 72$ h onwards). The experimental results demonstrated that Pd(II) was continuously removed from the aqueous phase by the biofilms as indicated by the decrease in Pd(II) concentration in the effluent and the linearly increasing amount of Pd immobilised in the biofilms. The removal of Pd(II) from the aqueous phase and the accumulation of Pd in the biofilms are shown in Figure 5.2.

Figure 5.2. Immobilisation of Pd in *S. oneidensis* MR-1 biofilms. Each data point represents the average of three replicates. Insets show the growth of *S. oneidensis* biofilms (pink color) in the first 3 days.

To check whether the accumulated Pd in the biofilms was Pd(0) resulted from Pd(II) reduction, the biofilms with 2-day exposure to Pd(II) were harvested and further characterized. A representative FE-SEM image and an EDX spectrum of the biofilms confirmed the presence of Pd precipitates in the biofilms (Figure 5.3A). The XRD spectrum of the biofilms showed peaks at around 40°, 46°, 68°, 82°, and 86° corresponding to the (111), (200), (220), (311), and (222) planes of an fcc lattice, respectively (card 05-0681 in the JCPDS file) (Figure 5.3B). The XRD pattern indicated that the Pd associated with the biofilms was in the form of Pd(0) crystals (De Windt, Boon et al. 2006).

Figure 5.3. (A) FE-SEM image and a representative EDX spectrum of the biofilm-Pd association; (B) XRD pattern of the biofilm-Pd association: peaks at around 40°, 46°, 68°, 82°, and 86° correspond to the (111), (200), (220), (311), and (222) planes of an fcc lattice, respectively.

We have shown that the biofilms reduced Pd(II) and accumulated Pd(0) crystals, but where did the Pd(0) crystals localise in the biofilms, i.e., in biofilm matrix or inside cells? We examined the resuspended biofilms using light microscopy by overlay of bright-field and fluorescence imaging of DAPI-stained cells (Figure 5.4A) and found a significant amount of extracellular nano-sized Pd(0) crystals in the biofilms, presumably immobilised by the biofilm matrix. The EPS matrix of *Shewanella biofilms* has been shown to contain many redox active components including electron carriers that could potentially reduce metal ions (Cao, Shi et al. 2011). The contribution of EPS matrix to the extracellular formation of uraninite nanoparticles during U(VI) reduction has been reported (Marshall, Beliaev et al. 2006, Cao, Ahmed et al. 2011, Cao, Shi et al. 2011). It is likely that certain redox components in the biofilm matrix might have contributed to Pd(II) reduction at the cell exterior.

Figure 5.4. (A) Dispersion of Pd(0) nanoparticles (black) in suspension of cells (green) from the biofilms visualised by overlay of bright-field and fluorescence imaging. Cells were stained with DAPI. For better image clarity, the image has been pseudo-colored. (B) TEM image showing the association of Pd(0) nanoparticles with bacterial cells at the single-cell scale. (C) Size distribution and zeta potential and (D) a representative TEM micrograph of the non-cell-associated Pd(0) nanoparticles in the biofilm-Pd(0) association.

The extracellular Pd(0) nanocrystals in the biofilms were separated from the bacterial cells and both fractions were further characterized. The cells from the biofilms were

examined by TEM at the single-cell level (Fig. 4B). Fine black (electron dense) nanoparticles (<20 nm) were found in the cell membrane while no nanoparticles were observed in the cytoplasm, suggesting an essential role of membrane-bound enzymes in Pd(II) reduction. Noted that the hydrogenase HyaB is in the periplasm, the formation of Pd(0) nanoparticles in the cell membrane could be, at least partially, due to the HyaB activity. The extracellular Pd(0) nanocrystals exhibited a narrow size distribution in the range of 8-50 nm with the majority has the diameter of 10-20 nm (Figure 5.4C and D). The zeta-potential of the particles was determined to be -33.5 ± 2.6 mV. High surface charge could be a possible reason why no large aggregates were found for these Pd(0) nanocrystals. Since many physicochemical properties such as catalytic activities of nanoparticles are size-dependent (Pal, Tak et al. 2007, Jiang, Kim et al. 2008, Park, Lim et al. 2011, Sobjerg, Lindhardt et al. 2011, Perez-Alonso, McCarthy et al. 2012), controlling the particle size in biosynthesis of nanoparticles is critically important. Our results demonstrated that the bioreductive formation of Pd(0) nanoparticles in the biofilms resulted in a relatively narrow particle size distribution, suggesting that biofilms could be a promising biocatalytic system for the synthesis of biogenic nanoparticles. Further, the Pd(0) nanoparticles with a narrow size distribution formed in biofilm matrix makes the biofilms a promising nanocatalytic system.

5.3.2. Biofilms with in situ generated Pd(0) nanocrystals have nanocatalytic activity

The catalytic activity of the two fractions of the biofilms, i.e., the extracellular matrix-associated Pd(0) nanoparticles and the cells (cell-Pd(0) association), was assessed by testing their activity in dechlorination reaction. Dechlorination rates of trichloroethylene (TCE) are shown in Figure 5.5.

Figure 5.5. TCE dechlorination rate by nanocatalytic biofilms and its various fractions, namely matrix-associated Pd(0) NPs and cell-associated Pd(0) NPs are shown in the graph. Pd(0)-free abiotic and biotic control were also conducted.

The Pd(0)-free abiotic and biotic control reaction has a dechlorination rate of 0.689 ± 0.31 $\mu\text{M/h}$ and 12.8 ± 0.71 $\mu\text{M/h}$, while TCE dechlorination rates for the reactions catalysed by the fraction of cell-associated Pd(0) NPs and the fraction of matrix-associated Pd(0) NPs were 18.9 ± 0.93 and 41.2 ± 0.32 $\mu\text{M/h}$, respectively. When both fractions are used

together, also termed nanocatalytic biofilm, the TCE dechlorination rate is 53.8 ± 1.21 $\mu\text{M/h}$. Table 5.2 shows the dechlorination rate of the nanocatalytic biofilm and its various fractions.

Table 5.2. The catalytic activity of the nanocatalytic biofilm and its two fractions of biofilms-associated Pd(0) nanoparticles are compared in the table.

	Rate of TCE Dechlorination ($\mu\text{mol TCE} / \text{mg catalyst} / \text{h}$)
Nanocatalytic biofilms	4.48
Cell-associated Pd(0) NPs	1.89
Matrix-associated Pd(0) NPs	20.59

Altogether, our results demonstrated that the biofilms with *in situ* formation of Pd(0) nanocrystals through bioreduction of Pd(II) were catalytically active and could be used as a novel biofilm-based biocatalysts. Further, our results reveal that, in addition to the cell-associated Pd(0) nanoparticles, the fraction of matrix-associated nanoparticles also showed high catalytic activity. Hence, effectively retaining nanoparticles could significantly improve the catalytic property of the biofilm-based biocatalysts, highlighting the critical role of EPS matrix in engineering biofilms for nanocatalysis.

5.3.3. Pd(II) inhibits cellular respiration and energy metabolism: risk of biofilm detachment

We have demonstrated the engineering of *S. oneidensis* biofilms for nanocatalysis via *in situ* bioreductive formation of Pd(0) nanocatalysts in biofilm matrix. Since Pd(II) is highly toxic (Melber, Keller et al. 2002), one important question would be how do the biofilms respond to Pd(II) during the engineering of biofilms for nanocatalysis. The answer may provide the basis for making the engineered biofilms more robust. To this end, we continuously monitored the dissolved oxygen concentration in the effluents at the outlet of the biofilm reactors (Figure 5.6).

Figure 5.6. A representative temporal profile of the dissolved oxygen concentration in the outlet of a biofilm reactor with *S. oneidensis* biofilms growing it.

Our results showed that the dissolved oxygen concentration in the outlet decreased with time and the dissolved oxygen was depleted on the third day (~52 h onwards), suggesting that the overall aerobic respiration activity of the biofilms was high and oxygen became a limiting nutrient from 52 h onwards. After Pd(II) was introduced into the biofilm reactors,

dissolved oxygen concentration in the effluent increased drastically from depleted oxygen condition to nearly inlet oxygen level within ~5 h, indicating an inhibition of the aerobic respiration of the biofilms by Pd(II).

To further elucidate the metabolic responses of *S. oneidensis* cells to Pd(II) exposure, iTRAQ-based quantitative proteomics analysis was carried out. Down-regulation of proteins involved in energy metabolism was observed in the presence of 1, 5 and 10 mM Pd(II) (Table 5.3). These proteins had annotated subrole functions in metabolism of amino acids and amines (IvdF, Ldh), electron transport (Azu, TrxA, SO4048, MtrA, PetA, CytC), anaerobic energy metabolism (NrfA), and central intermediary metabolism (Icd, Fbp, Pfk). The down-regulation of these proteins, especially the electron carriers involved in the electron transport, suggest a reconfiguration of energy metabolism by the cells in response to Pd(II) exposure, which is consistent with the observation of significant inhibition in aerobic respiration. The down-regulation of these energy metabolism proteins was dosage-dependent with a general trend of more repression in the presence of higher concentration of Pd(II).

Table 5.3. Selected down-regulated proteins involved in energy metabolism.

Locus tag	Gene	Protein description	#Peptides / Coverage	1:0	5:0	10:0
SO0406	<i>trxA</i>	Thioredoxin 1 TrxA	14 / 82.4%	0.17	0.17	0.12
SO0608	<i>petA</i>	Ubiquinol-cytochrome c reductase FeS subunit PetA	5 / 33.7%	0.24	0.13	0.04
SO0809	<i>azu</i>	Periplasmic azurin Azu	3 / 34.8%	0.08	0.21	0.20
SO0932	<i>pgk</i>	Phosphoglycerate kinase Pgk	16 / 51.2%	0.29	0.15	0.14
SO1682	<i>ivdF</i>	3-hydroxyisobutyrate dehydrogenase IvdF	5 / 23.7%	0.24	0.10	0.22
SO1777	<i>mtrA</i>	Periplasmic decaheme cytochrome c component MtrA	2 / 11.4%	0.40	0.25	0.04
SO2629	<i>icd</i>	Isocitrate dehydrogenase NADP-dependent Icd	17 / 39.4%	0.46	0.22	0.06

SO2638	<i>ldh</i>	Leucine dehydrogenase Ldh	19 / 61.9%	0.47	0.15	0.06
SO3980	<i>nrfA</i>	Ammonia-forming nitrite reductase NrfA	11 / 39%	0.24	0.28	0.06
SO3991	<i>fbp</i>	Fructose-16-bisphosphatase Fbp	3 / 20.9%	0.15	0.28	0.44
SO4048	-	Diheme cytochrome c4	4 / 28.9%	0.22	0.09	0.06
SO4666	<i>cytcB</i>	Diheme cytochrome c4 CytC	5 / 45.4%	0.13	0.09	0.03

The inhibition of oxygen respiration and down-regulation of energy metabolism of the cells exposing to Pd(II) suggest that the cells could obtain only significantly less metabolic energy comparing to those prior to Pd(II) exposure. The stability of *S. oneidensis* biofilms has been shown to be energy-dependent and metabolic energy is required for maintaining cell attachment (Saville, Rakshe et al. 2011). Inhibition of energy metabolism could lead to biofilm dispersal. We quantified cell detachment from the biofilms before and after Pd(II) exposure and found that the detachment rate after 12 h exposure to Pd(II) was $2.07 \pm 0.26 \times 10^9$ cells/h/cm², which was significantly higher than that prior to Pd(II) exposure ($1.30 \pm 0.11 \times 10^9$ cells/h/cm²). The results implied the risk of biofilm detachment during engineering biofilms for nanocatalysis via exposure to Pd(II). A comprehensive understanding of biofilm detachment will be required towards the development of robust biofilm-based biocatalysts for continuous bioprocesses, which warrants further investigation.

5.3.4. Genetic engineering of intracellular c-di-GMP for robust nanocatalytic biofilm

A novel method and a comprehensive understanding of biofilm detachment will be required towards the development of robust biofilm-based biocatalysts for continuous bioprocesses. To this end, we deployed genetic engineering to alter the intracellular c-di-GMP level to influence biofilm-related processes for better biofilm performance. Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) functions as an important second messenger in microbial systems (Hengge 2009). C-di-GMP is synthesized by diguanylate cyclases (containing a GGDEF domain) and degraded by phosphodiesterase. There had been previous reports that c-di-GMP influence the switching of sessile biofilm and motile planktonic mode of life in many microbes,

stimulating the secretion of various EPS components, and affect their motility and virulent capabilities (Gjermansen, Ragas et al. 2006, Jenal and Malone 2006, Wolfe and Visick 2008, Broberg, Calder et al. 2011, Spurbeck, Tarrien et al. 2012). Hence, we feel that c-di-GMP is a suitable candidate for this study as a regulator of the formation and detachment of microbial biofilm.

5.3.5. c-di-GMP quantification in MR-1 WT vs c-di-GMP mutant

To determine whether c-di-GMP mutant produce higher amount of cytoplasmic c-di-GMP as compared to the WT, we quantified the amount of cytoplasmic c-di-GMP using LC with commercial c-di-GMP standards. We normalised the amount of c-di-GMP by the amount of cytoplasmic proteins to prevent any discrepancy arise due to cell lysing efficiency. We found that c-di-GMP mutant has ~10 times higher cytoplasmic c-di-GMP level compared to WT. This verifies that we are able to produce c-di-GMP mutant that indeed has higher level of c-di-GMP in the cytoplasm via insertion of *pYedQ2* into the *S. oneidensis* MR-1.

5.3.6. Transcriptomic analysis: Influence of increasing c-di-GMP on Mtr pathway in MR-1

In order to shed light on the physiological difference between *S. oneidensis* MR-1 WT and c-di-GMP mutant, total RNA from the cells was extracted from 8-hour mid log-phase cultures of the respective strains. There were 444 genes that were expressed significantly different at the transcript level in the c-di-GMP mutant compared to the WT. Among those up-regulated in c-di-GMP mutant, we found that the all the genes encoding the five primary protein components of MTR pathway have higher expression at the transcript level in c-di-GMP mutant compared to the WT, as shown (Table 5.4).

Table 5.4. Transcriptomic data of MTR-pathway-related gene expression in *S. oneidensis* MR-1 WT and c-di-GMP mutant.

gene symbol	locus tag	gene description	gene ontology	normalised p-value	log2 ratio
omcA	SO_1779	extracellllular iron oxide respiratory system surface decaheme cytochrome c component OmcA	electron carrier activity	2.244E-12	3.728
mtrC	SO_1778	extracellular iron oxide respiratory system surface decaheme cytochrome c component MtrC	electron carrier activity	6.693E-10	2.796
mtrA	SO_1777	extracellllular iron oxide respiratory system periplasmic decaheme cytochrome c component MtrA	electron carrier activity	3.203E-10	2.241

cymA	SO_4591	membrane anchored tetraheme cytochrome c CymA	electron carrier activity, nitrate reductase activity, succinate dehydrogenase activity	1.165E-08	2.202
mtrB	SO_1776	extracellular iron oxide respiratory system outer membrane component MtrB		1.489E-09	1.684

The Mtr pathway consists of 5 primary protein components, namely OmcA, MtrC, MtrB, MtrA and CymA. It is one of the most well-studied extracellular electron transport chain modules in *S. oneidensis* MR-1, and plays an important role on cellular respiration using mineral oxides, e.g. Hematite, as extracellular terminal electron acceptors (Ng, Cai Tan et al. 2013). The MTR pathway is also important in the area of bioremediation, specifically in the conversion of environmental contaminants to an insoluble and less harmful form. Examples include reduction of Cr(VI) to Cr(III) (Belchik, Kennedy et al. 2011), U(VI) to U(IV) (Lovley, Phillips et al. 1991), $\text{Tc(VII)O}_4^-(\text{aq})$ to $\text{Tc(IV)O}_2(\text{s})$ (Marshall, Plymale et al. 2008) and Ag^+ to Ag(0) (Ng, Sivakumar et al. 2013) etc. The MTR pathway, which confer *S. oneidensis* MR-1 its ability to perform extracellular electron transfer, is also essential in electricity generation in microbial fuel cells (Harris, El-Naggar et al. 2012, Okamoto, Hashimoto et al. 2012).

With the increase in gene expression of MTR pathway-related genes at transcript level, we are interested to know whether there are any increases in amount of functional MTR pathway protein and/or changes in the effectiveness of the MTR pathway. We performed Fe(III) reduction using resting cells assay, and found that under similar condition, c-di-GMP mutant is able to reduce Fe(III) about two time faster compared to the WT (Figure 5.7).

Figure 5.7. Rate of Fe(III) reduction by *S. oneidensis* MR-1 WT and c-di-GMP mutant.

This shows that there are either more functional MTR proteins in c-di-GMP mutant, which allow for higher rate of extracellular electron transfer per cell, or the MTR proteins are functioning at a higher efficiency.

To further verify this observation, we performed western blot to determine the relative amount of MtrC and OmcA proteins in WT and c-di-GMP mutants, using MR-1 mutant strain lacking gene encoding the protein MtrC and OmcA, i.e. $\Delta mtrC$ and $\Delta omcA$, as a negative control. We found that there is indeed an increase amount of outer membrane c-type cytochrome MtrC and OmcA in the c-di-GMP mutant as compared to the WT, as shown (Figure 5.8).

Figure 5.8. Western Blot: Quantification of outer-membrane c-type cytochrome MtrC and OmcA in *S. oneidensis* MR-1 WT and c-di-GMP, with mutant lacking MtrC (e.g. $\Delta mtrC$) and OmcA (e.g. $\Delta omcA$) as negative control, in the form of (A) Gel image (B) Bar chart.

5.3.7. Transcriptomic analysis: Influence of increasing c-di-GMP on Motility in MR-1.

We found that 21 genes encoding motility-related proteins are significantly down-regulated in c-d-GMP mutant as compared to the WT (Table 5.5). This suggests that an increase in the level of cytoplasmic c-di-GMP leads to a decrease in cellular motility in *S. oneidensis* MR-1.

Table 5.5. Transcriptomic data of motility-related gene expression in *S. oneidensis* MR-1 WT and c-di-GMP mutant.

Gene symbol	Locus tag	Gene description	Gene ontology	Normalised p-value	Log2 ratio
<i>flhA</i>	SO_3213	flagellar export protein FlhA	ciliary or bacterial-type flagellar motility	1.246E-06	-2.243
<i>flgE</i>	SO_3247	flagellar hook protein FlgE	structural molecule activity, ciliary or bacterial-type flagellar motility	5.027E-08	-2.369
<i>fliS</i>	SO_3233	chaperone for FliC flagellin FliS	ciliary or bacterial-type flagellar motility	3.302E-06	-2.373
<i>flgG</i>	SO_3244	flagellar component of cell-distal portion of basal-body rod FlgG	structural molecule activity, ciliary or bacterial-type flagellar motility, bacterial-type flagellum basal body	3.655E-06	-2.418

<i>flgI</i>	SO_3242	flagellar P-ring protein FlgI	structural molecule activity, ciliary or bacterial-type flagellar motility	1.252E-04	-2.495
<i>flgL</i>	SO_3239	flagellar hook-associated protein FlgL	structural molecule activity, ciliary or bacterial-type flagellar motility	1.867E-05	-2.500
<i>flrB</i>	SO_3231	two component signal transduction system controlling flagellar biosynthesis histidine kinase with PAS sensory domain FlrB	phosphorelay sensor kinase activity, protein kinase activity, ciliary or bacterial-type flagellar motility, phosphorelay signal transduction system	2.241E-06	-2.613
<i>flgC</i>	SO_3249	flagellar basal-body rod protein FlgC	structural molecule activity, ciliary or bacterial-type flagellar motility	4.951E-05	-2.710

<i>fliC</i>	SO_3238	flagellin FliC	structural molecule activity, ciliary or bacterial-type flagellar motility	1.446E-10	-2.735
<i>fliK</i>	SO_3223	flagellar hook-length control protein FliK	ciliary or bacterial-type flagellar motility	1.282E-09	-2.845
<i>motY</i>	SO_2754	smf-dependent flagellar motor protein MotY	motor activity, ciliary or bacterial-type flagellar motility	2.160E-06	-2.967
<i>fliC</i>	SO_3237	flagellin FliC	structural molecule activity, ciliary or bacterial-type flagellar motility	3.249E-11	-3.043
<i>flgJ</i>	SO_3241	flagellar rod cap protein and peptidoglycan hydrolase FlgJ	catalytic activity, ciliary or bacterial-type flagellar motility, outer membrane-bounded periplasmic space	9.000E-08	-3.235

<i>flgD</i>	SO_3248	flagellar hook assembly protein FlgD	ciliary or bacterial-type flagellar motility	2.906E-08	-3.264
<i>flgM</i>	SO_3254	flagellar biosynthesis anti-sigma factor FlgM	ciliary or bacterial-type flagellar motility, regulation of transcription, DNA-dependent	2.906E-08	-3.398
<i>flgB</i>	SO_3250	flagellar basal-body rod protein FlgB	structural molecule activity, ciliary or bacterial-type flagellar motility	1.552E-07	-3.439
<i>flgN</i>	SO_3255	secretion chaperone for FlgK and FlgL FlgN	ciliary or bacterial-type flagellar motility	3.897E-09	-3.494
<i>fliD</i>	SO_3235	flagellar filament capping protein FliD	structural molecule activity, ciliary or bacterial-type flagellar motility	1.704E-12	-3.520

<i>flgF</i>	SO_3245	flagellar component of cell-proximal portion of basal-body rod FlgF	structural molecule activity, ciliary or bacterial-type flagellar motility	2.025E-09	-3.541
<i>fliF</i>	SO_3228	flagellar basal-body MS-ring and collar protein FliF	structural molecule activity, ciliary or bacterial-type flagellar motility, bacterial-type flagellum basal body	2.078E-10	-3.544
<i>fliE</i>	SO_3229	flagellar basal-body component FliE	structural molecule activity, ciliary or bacterial-type flagellar motility	1.503E-05	-3.570

With this hypothesis in mind, we proceed with swimming motility test using LB plate with 0.3% agar to determine if the swimming motility of the c-di-GMP mutant is really compromised. We found that colonies formed from c-di-GMP mutant have an average of about 40% shorter diameter compared to those from WT, as shown (Figure 5.9).

Figure 5.9. Swimming Motility Assay: Comparison of colony diameter between *S. oneidensis* MR-1 WT and c-di-GMP mutant.

There have been many reports on the influence of c-di-GMP on cellular motility. *Vibrio cholerae* uses its transcriptional regulator VpsT to directly sense c-di-GMP and regulate cell motility. The sensing is done by a change in oligomerisation of VpsT when it binds to c-di-GMP (Krasteva, Fong et al. 2010). In *E. coli* and *Salmonella*, a c-di-GMP binding protein YcrG interacts with flagella switch-complex proteins to induce motility inhibition by reducing the torque generated by the flagella in the presence of c-di-GMP (Paul, Nieto et al. 2010). In *Pseudomonas aeruginosa* PA14, *bifA* gene

(PA4367) encodes both GGDEF and EAL domains. Deletion of the *bifA* gene leads to increased cellular c-di-GMP level and a severe defect in swarming motility compared to PA14 WT (Kuchma, Brothers et al. 2007). Our result is in line with previous literatures which indirectly proposed a similar trend in *S. oneidensis* MR-1 (Thormann, Duttler et al. 2006, Rakshe, Leff et al. 2011, Chao, Rakshe et al. 2013). However, when coupled with the quantification of cytoplasmic c-di-GMP level, this study validates directly for the first time that an increase in the level of cytoplasmic c-di-GMP leads to a decrease in swimming motility in *S. oneidensis* MR-1.

5.3.8. Influence of increasing c-di-GMP on biofilm formation and detachment in MR-1.

C-di-GMP mutant appears to have a stronger binding adhesiveness as compared to WT, which was suggested by the formation of biofilm (cell-solid substratum adhesion) in planktonic culture. It is also observed that c-di-GMP mutant biofilm has much higher viscosity compared to WT (Figure 5.10).

Figure 5.10. Viscosity measurement of *S. oneidensis* MR-1 WT and c-di-GMP mutant biofilm.

To determine whether biofilm formation is affected by the increase in cytoplasmic c-di-GMP, we grow *S. oneidensis* MR-1 WT and c-di-GMP mutant in a 96-well plate and flow cell system to examine static and hydrodynamic biofilm growth respectively. Static biofilm assay is a high throughput method to screen bacteria according to their biofilm-forming capability in a hydrostatic environment. In this study, we measured the biofilm formation and amount of planktonic cells in 96 well-plates, and observed higher biofilm growth and lower amount of planktonic cells in c-di-GMP mutant when compared to WT, as shown (Figure 5.11). The total amount of cells in each well (planktonic and biofilm) was quantified via CFU, which showed no significant difference between WT and c-di-GMP mutant.

Figure 5.11. OD₆₀₀ measurement (left y-axis) and static biofilm assay (right y-axis), showing the relative amount of planktonic cells and biofilm of *S. oneidensis* MR-1 WT and c-di-GMP mutant, respectively.

We then proceeded with the flow cell biofilm experiment and found that c-di-GMP mutant formed thicker biofilms (Figure 5.12). In a tubing biofilm set-up, we found that c-di-GMP has much lower biofilm detachment rate compared to WT, as shown (Table 5.6).

Figure 5.12. Flow cell biofilm: CLSM 3D images and orthogonal views of *S. oneidensis* MR-1 WT (A and C) and c-di-GMP mutant (B and D) respectively, showing biofilm formation after 72 hours of growth under hydrodynamic conditions. The white bar represents 100 μm.

Table 5.6. Rate of cell detachment from flow cell biofilms of *S. oneidensis* MR-1 WT and c-di-GMP mutant.

	Cell detachment rate (cells h ⁻¹ mg ⁻¹)			
	60 th hour	66 th hour	78 th hour	84 th hour
MR-1 WT	$1.8 \pm 0.24 \times 10^8$	$1.7 \pm 0.37 \times 10^8$	$3.2 \pm 0.26 \times 10^8$	$3.1 \pm 0.46 \times 10^8$
MR-1 c-di-GMP mutant	$3.2 \pm 0.35 \times 10^7$	$3.1 \pm 0.38 \times 10^7$	$5.5 \pm 0.31 \times 10^7$	$5.2 \pm 0.62 \times 10^7$

We isolated the biofilm and measured the dry biomass, and found that the biomass of WT and c-di-GMP is in the ratio of 1:15 respectively. This difference in amount of cell mass between WT and c-di-GMP biofilm under hydrodynamic condition is much more prominent than in the static biofilm set-up. This difference, coupled with the lower cell detachment rate in c-di-GMP mutant biofilm compared to WT, suggests that biofilm containing cells with higher c-di-GMP level are able to resist detachment and are less adversely affected by the toxicity of Pd(II) ions.

Our results show for the first time that biofilm formation in *S. oneidensis* MR-1 can be promoted via insertion of plasmid carrying the *yedQ* gene to elevate the production of c-di-GMP in the cytoplasm. This is an important step forward, especially in the area of bioremediation and biofilm-related industrial processes. The ability to form robust biofilms is essential for processes that occur in hydrodynamic environment, such as bioremediation of a contaminated site or biosynthesis of fine chemicals in a continuous-mode industrial reactor. There are many bacteria that can convert contaminants to benign substances, but are impractical to use in bioremediation due to weak attachment to the contaminated surface, short active period and low tolerance to the concentration of contaminant. The ability to genetically engineer the bacteria to form biofilm more readily, via e.g. insertion of *pYedQ2*, will make it more feasible to use those bacteria for bioremediation purposes, and lowers the criteria to allow a wider variety of bacteria to be used for the same.

5.3.9. Influence of increasing c-di-GMP on activity of nanocatalytic biofilm.

Biofilms of MR-1 wt and c-di-GMP mutant were used to produce Pd NPs after three days of growth. The resultant nanocatalytic biofilm of MR-1 wt and c-di-GMP,

normalized by biomass, were test for their activity using Chromium(VI) reduction as model reaction as shown in Figure 5.13.

Figure 5.13. Percentage of Cr(VI) removed by various biofilm samples, namely biofilm control (no Pd) of wt and c-di-GMP mutant and nanocatalytic biofilm wt and c-di-GMP mutant in the first and fifth reaction with catalyst recycling. All experiments are done in triplicates.

There are relatively low Cr(VI) removal by the biofilm control (no Pd) of both WT and c-di-GMP mutant, where the amount of Cr(VI) removed is around 20% and 25% respectively. Cr(VI) removal in nanocatalytic biofilm of wt and c-di-GMP mutant were relatively similar, where the amount of Cr(VI) removed is around 69% and 72% respectively. Cr(VI) reduction was repeated five times using the same samples, namely biofilm control (no Pd) of wt and c-di-GMP mutant and nanocatalytic biofilm wt and c-di-GMP mutant. It was observed that the amount of Cr(VI) removed across all samples decreased significantly. Interestingly, amount of Cr(VI) removed for

nanocatalytic biofilm wt decreased by 74% while that of nanocatalytic biofilm c-di-GMP mutant decreased only by 43%. This shows that although nanocatalytic biofilm wt and c-di-GMP mutant have similar activity, the c-di-GMP mutant showed much lower loss of activity over multiple reactions as compared to wt. This can be attributed to the lower cell detachment rate in nanocatalytic biofilm c-di-GMP mutant, and hence a slower rate of Pd leaching as compared to wt.

5.4 Conclusions

In this study, we show that (i) the *S. oneidensis* biofilms reduce Pd(II) and accumulate Pd(0) nanocrystals with a size of 10-20 nm in the biofilm matrix and in the cell membrane even under aerobic conditions; (ii) the *S. oneidensis* biofilms with Pd(0) nanocrystals formed and immobilised *in situ* in the biofilms exhibit nanocatalytic activity; (iii) Pd(II) exposure inhibits cellular respiration and energy metabolism of *S. oneidensis* cells, posing a risk of biofilm detachment; (iv) increasing the intracellular level of cyclic-di-GMP in *S. oneidensis* MR-1 can reduce the biofilm detachment; and (v) c-di-GMP is an upstream signalling molecule which influence several key cellular processes, such as cellular motility, extracellular electron transport, iron uptake and biofilm formation in *S. oneidensis* MR-1. Our results suggest a novel strategy for engineering biofilms towards the development of biofilm-based multifunctional biocatalysts for continuous bioprocesses. Since the formation of nanocatalysts in biofilms is a mild and environment-friendly biological process in aqueous media, the strategy of engineering biofilms for nanocatalysis also reflects the sustainable development in chemical industry.

CHAPTER 6: SYNTHESIS OF ROBUST BIOFILM-TEMPLATED HETEROATOM-DOPED CARBON-PALLADIUM NANOCOMPOSITE CATALYST FOR HEXAVALENT CHROMIUM REDUCTION

A portion of this chapter has been presented in oral format as Ng CK and Cao B (2016). Engineering biofilm for remediation of environmental chromium(VI) contamination. 6th International Conference on Environment Science and Biotechnology (ICESB 2016), Kyoto, Japan.

6.1. Introduction

Chromium is commonly used in various industries, such as electroplating, mining and hydrocarbon processing (Barnhart 1997). Its widespread use has contributed to its release via wastewater into the environment, mainly in the form of trivalent chromium [Cr(III)] and hexavalent chromium [Cr(VI)] (Costa 2003). Cr(VI), mainly in the form of $\text{Cr}_2\text{O}_7^{2-}$ and CrO_4^{2-} , is toxic and hazardous to living organisms and can cause serious consequences to aquatic ecosystems due to its high water solubility and mobility in the environment (Leonard and Lauwerys 1980, Katz and Salem 1993, O'Brien, Ceryak et al. 2003, Costa and Klein 2006, Salnikow and Zhitkovich 2008). Cr(VI) can cause chronic health problems to human and is classified as a group 1 carcinogen by the International Agency for Research on Cancer (International Agency for Research on Cancer 1987). On the other hand, Cr(III) is much less toxic than Cr(VI), has low solubility in water under circumneutral pH, and can be easily removed from the environment through precipitation or adsorption on various adsorbents (Belchik, Kennedy et al. 2011, Ali 2012, Chebeir and Liu 2016). As such, it has been suggested that effective and efficient remediation of Cr(VI) can be achieved via the reduction of

Cr(VI) to the less toxic Cr(III), followed by its adsorption and/or precipitation (Blowes, Ptacek et al. 1997, Ponder, Darab et al. 2000, Chai, Huang et al. 2009).

Current strategies for the reduction of Cr(VI) to Cr(III) involves electro-reduction, chemical reduction, photocatalytic reduction and biological reduction (Lai and Lo 2008, Wang, Wang et al. 2008, Huang, Ma et al. 2012, Ding, Peng et al. 2013). For instance, Nazuhah and Hu et al developed a plant-microbial fuel cell for the reduction of Cr(VI) to Cr(III), and found that both the Cr(VI) removal rates and the removal efficiencies increased with the increasing initial Cr(VI) concentration (Habibul, Hu et al. 2016). Lai et al demonstrated Cr(VI) bioreduction using methane (CH₄) as the sole electron donor in a membrane biofilm reactor, and Cr(VI) removal at the end of the 90-day test reached 95% at an influent Cr(VI) concentration of 3 mg Cr/L and a surface loading of 0.37 g of Cr m⁻² day⁻¹ (Lai, Zhong et al. 2016).

A hybrid between biological and chemical reduction approaches, biogenic noble metal nanoparticles (NPs) have gained increasing attention as a viable environmental catalyst suitable for Cr(VI) reduction (Hennebel, De Gusseme et al. 2009, Gadd 2010, De Corte, Hennebel et al. 2011, Lloyd, Byrne et al. 2011, De Corte, Hennebel et al. 2012, Hennebel, De Corte et al. 2012, Hosseinkhani, Hennebel et al. 2014). Zero-valent palladium is a commonly used catalyst for the reduction of Cr(VI), while low cost electron donors such as hydrogen (H₂) and formate ions can be used to facilitate the reduction process (Omole, K'Owino et al. 2007, Chidambaram, Hennebel et al. 2010). Biogenic palladium nanoparticles (Pd NPs) that are produced from bacteria, such as *S. oneidensis* MR-1, have high catalytic properties (De Windt, Boon et al. 2006, Hennebel, Simoen et al. 2009, Hennebel, De Corte et al. 2010, De Corte, Hennebel et al. 2011, De Corte, Sabbe et al. 2012). However, metal NPs synthesized from

planktonic bacteria may be subjected to leaching and the bacteria cells may lose viability due to high toxicity from the metal ions (Ng, Cai Tan et al. 2013, Ng, Sivakumar et al. 2013). Several studies have pointed to biofilm, a structured, surface-associated, microbial community, as a potential biomaterial and structural support for the synthesis of metal NPs, with benefits such as reduced leaching of metal nanoparticles, increased tolerance to toxic metal ions etc (Gross, Hauer et al. 2007, Gross, Lang et al. 2010, Halan, Buehler et al. 2012). There have also been studies which suggested that metal nanoparticles formed on mesoporous structures may exhibit increased catalytic activity and long-term stability (Matveeva, Valetsky et al. 2011, Scirè and Liotta 2012, Yates, Cusick et al. 2014). It is important to explore various methods that may potentially increase the efficiency and effectiveness of Cr(VI) reduction.

In this study, we first synthesized Pd NPs on living *S. oneidensis* MR-1 biofilm supported on inert carbon felt. The nano-Pd biofilm were then subjected to controlled pyrolysis to obtain a mesoporous carbon-palladium (C-Pd) nanocomposite. The nano-Pd biofilm and the C-Pd nanocomposite were then characterized and tested for their catalytic activity on the reduction of Cr(VI). The reusability of both catalytic systems was also evaluated. To the best of our knowledge, there have been no studies to characterize and compare the catalytic activities and the robustness of palladium nanoparticles synthesized on living biofilm and pyrolysed mesoporous carbon support for the reduction of Cr(VI) to Cr(III) in an aqueous environment. This study demonstrated for the first time the sustainable engineering of biofilms for nanocatalytic activity through *in situ* formation of nanocatalysts in biofilm matrix and influencing the robustness of the catalyst via controlled pyrolysis for the application of environmental contaminant removal.

6.2. Materials and Methods

6.2.1. Chemicals, Bacterial strains and growth conditions

Na₂PdCl₄, K₂Cr₂O₇, Pd nanopowder (<25 nm particle size (TEM), ≥99.5% trace metals basis) and all other chemicals were purchased from Sigma Aldrich and were used without further purification. Stock cultures *S. oneidensis* MR-1 wild-type (WT) and high c-di-GMP strain *pYedQ₂* were maintained in LB medium with 20% glycerol at -80°C. The bacterial cells were grown in LB medium or modified M1 medium under aerobic or anaerobic conditions. The modified M1 medium (pH ~7.0) consisted of 3.00 mM PIPES, 7.50 mM NaOH, 26.04 mM NH₄Cl, 1.34 mM KCl, 4.35 mM NaH₂PO₄ and 0.68 mM CaCl₂ supplemented with trace amounts of minerals, vitamins, and amino acids (Cao, Shi et al. 2011). For bacterial growth in M1 medium, 20 mM sodium lactate or sodium formate was used as an electron donor and the sole carbon source. For growth under anaerobic conditions, the medium containing 10 mM ferric nitrilotriacetate (Fe(III)-NTA) as an electron acceptor was bubbled with nitrogen gas for 5 min and the headspace in the culture was less than 3% of the total volume.

6.2.2. Growth of *S. oneidensis* biofilms and Pd(II) reduction

Three replicates of submerged biofilm reactors were set up using 3-ml syringes (Luer-Lok tip, latex free; BD) loosely packed with carbon felt (3.18mm thick, 99%; VWR, Singapore) for biofilms to attach and grow using modified M1 medium containing 10 mM formate continuously supplied through a peristaltic pump at a flow rate of 8 ml/h. The biofilm reactor system was assembled and prepared as described previously (Ding, Peng et al. 2013). Each biofilm reactor was inoculated by injecting 2 ml overnight culture (diluted to an OD₆₀₀ of 0.1) using a syringe. After inoculation, the

medium flow was stopped for 1 h to allow initial attachment followed by continuous media flow with a flow rate of 8 ml/h.

The *S. oneidensis* biofilms were allowed to grow for 72 h, and then the feed medium was loaded with 1 mM Pd(II). Effluent samples were collected and the concentration of Pd(II) was determined using the colorimetric method described previously (Rotaru, Jiang et al. 2012). The same setup with no biofilms growing in it served as a control. The amount of Pd accumulated in the biofilms was estimated by subtracting the total amount of Pd(II) introduced into the biofilm reactors by the amount of Pd(II) remained in the effluents. Samples from the media effluents were taken periodically and cell detachment was evaluated by counting colony forming unit (CFU) using the drop-plate method described elsewhere (Chen, Nace et al. 2003).

6.2.3. Field emission scanning electron microscopy and energy-dispersive X-ray spectroscopy

Samples were re-suspended in MilliQ water. A portion of each suspension was dried under vacuum and then coated with platinum using a vacuum electric sputter coater (JEOL JFC-1300, JEOL Asia Pte. Ltd., Singapore). SEM images were then taken using a Field Emission Scanning Electron Microscope (FE-SEM, JSM-7600, JEOL Asia Pte. Ltd., Singapore) at a voltage of 2.0-5.0 kV. EDX spectrum was obtained using an energy-dispersive X-ray spectroscope (AZtecEnergy, Oxford Instruments, Oxfordshire, UK).

6.2.4. Controlled pyrolysis of biofilm-nanopalladium catalyst

Biofilms supported with carbon fiber felt substrates were pyrolysed yielding C-Pd nanocomposite catalysts. Typically, samples were put in ceramic boats and were

subjected to 850 °C inside sealed quartz tubes under the flow of ultrapure argon (20 sccm, 99.9999%, Soxal). Heating profile of carbonisation process was as follows: 1) heating with ~1 °C/min from ambient temperature up to 850 °C, 2) with isothermal hold at 850 °C for 4 h, and 3) cooling down to room temperature under argon stream.

6.2.5. X-ray diffraction

To further characterize the Pd associated with the biofilms before and after pyrolysis, X-ray diffraction (XRD) analysis of the dried samples was performed using a Bragg-Brentano diffractometer (Bruker D8 Advanced) in θ - 2θ configuration with a Cu K α line at 0.15418 nm as a source generated by a copper X-ray tube with power 2.2 kW (40 kV, 40 mA). Measurements were performed in the range of $2\theta = 20$ -90 degrees.

6.2.6. Chromium reduction of the catalytic systems

Samples testing for chromium reduction activity was added into a 50 ml eppendorf tube containing 25 ml of the following reaction media: 1% formic acid and 0.05 mM potassium dichromate ($K_2Cr_2O_7$), i.e., 0.1 mM Cr(VI), in HEPES buffer (30 mM, pH 7.0). The reaction system was incubated at room temperature on a shaker (200 rpm) in the dark. The concentration of Cr(VI) was determined using a previously reported s-diphenylcarbazide colorimetric method (Guha, Jayachandran et al. 2001). Briefly, a 0.8 ml sample was mixed with 0.1 ml of 0.5 g/liter s-diphenylcarbazide in 10% methanol-12.5 mM H_2SO_4 followed by OD₅₄₀ measurement after a 20-min incubation. The presence of Cr(III) as the reaction product was confirmed using sodium and ammonium hydroxide. An excess of sodium hydroxide solution was added to a solution of Cr(III) ions, a green solution of hexahydroxochromate(III) ions was produced confirming the presence of Cr(III).

6.3. Results and Discussion

6.3.1. Formation of abiotic biofilm-templated heteroatom (N and P)-doped carbon-palladium nanocomposite via controlled pyrolysis

S. oneidensis MR-1 biofilm can reduce Pd(II) and immobilise Pd(0) nanoparticles on the cell surface and within the EPS matrix under aerobic conditions (Figure 6.1).

Figure 6.1. Schematic diagram of (A) functionalising biofilm with Pd NPs, resulting in nanocatalytic biofilm and (B) possible interaction between Pd NPs and EPS components.

However, toxic Pd(II) exposure leads to biofilm detachment, where loss of biomass and potential leeching of Pd nanoparticles may result. While genetic manipulation can partially overcome this problem, maintenance of biotic nanocatalytic biofilm in term of nutrient uptake and controlling biofilm-related processes (e.g. natural biofilm dispersal) are still some of the major barriers which prevent the widespread use of biofilm-based catalyst for large-scale industrial applications. A novel method is needed to engineer the biofilm-based catalyst that is robust and feasible for large-scale industrial use. As such we propose the pyrolysis of the nanocatalytic biofilm to form an abiotic catalyst which might alleviate the bottlenecks of a biotic catalyst.

Previous study has suggested that biotemplated palladium catalysts can be stabilised on support materials (Yates and Logan 2014). In this study, carbon felt was selected to be the support material as it is inert to both biofilm growth and pyrolysis treatment. To prevent the leeching of Pd from the biofilm through cell detachment and drastically improve the robustness of the catalyst, we attempted controlled pyrolysis of the biofilm-nanopalladium catalyst of MR-1 c-di-GMP mutant to form abiotic C-Pd nanocomposite using carbon felt as support material (Figure 6.2).

Figure 6.2. SEM image of (A) carbon felt, (B) biofilm-nanopalladium catalyst on carbon felt, (C) pyrolysed biofilm-templated carbon-palladium nanocomposite on carbon felt. White bar represents 1 μm . Images are taken with the same magnification (x 5000). SEM-EDX image of (D) an area of interest of the biofilm-templated C-Pd nanocomposite, and EDX-profile of the area of interest of (E) palladium, (F) phosphorus and (G) nitrogen

XRD measurement on the C-Pd nanocomposite confirmed the presence of crystalline Pd, while SEM-EDX revealed the presence of palladium, phosphorus and nitrogen on the C-Pd composite (Figure 6.2E, F and G). Phosphorus and nitrogen were not present in the original carbon felt, which indicates that biofilm is the sole source of heteroatoms (N and P) found in the pyrolysis product. Phosphorus and nitrogen can be found naturally in most, if not, all bacterial biofilms. Main sources of phosphorus are extracellular DNA (eDNA) and cell membranes, while main sources of nitrogen are polypeptides (Flemming and Wingender 2010).

EDX image showed that palladium tends to form nanoparticles (Figure 6.2E) while phosphorus and nitrogen are evenly distributed over the sample area (Figure 6.2F and G). The SEM image of the C-Pd nanocomposite showed a rough, porous structure on the surface of the carbon felt (Figure 6.2C and D). Together, these findings may suggest that the C-Pd nanocomposite coated evenly throughout the carbon felt and the palladium retained nanocrystalline structure after pyrolysis. The carbonized biofilm form mesoporous nanocomposite structures with high surface to volume ratio, possibly improving the catalyst's functionality to remediate Cr(VI).

6.3.2. Cr(VI) reduction and catalyst recycling

We assessed the Cr(VI) reduction ability of the pyrolyzed biofilm with and without palladium, along with non-pyrolyzed biofilm-nanoPd catalyst and biofilm-only samples using 0.1 mM of Cr(VI) with 1% formic acid as electron donor over 10 minutes of reaction. We found that the Cr(VI) reduction ability of the C-Pd nanocomposite is significantly much higher than biofilm-nanoPd and biofilm samples (Figure 6.3). Formic acid alone cannot reduce Cr(VI) under our experimental condition.

Figure 6.3. Cr(VI) reduction of C-Pd nanocomposite, nanocatalytic biofilm (c-di-GMP strain), no-Pd biofilm control (c-di-GMP) and pyrolyzed biofilm control (no Pd) samples on the 1st and 5th catalytic reaction. Initial concentration of 0.1 mM Cr(VI) was introduced into the system, and formic acid (1%) was used as electron donor. All Cr(VI) reduction experiment are conducted in triplicates.

The no-Pd biofilm control (c-di-GMP strain) is able to remove around 20% of the Cr(VI), which is due to the Cr(VI) bioreduction ability of *S. oneidensis* MR-1 bacteria and also the adsorption of Cr(VI) within the biofilm matrix, which was shown in previous studies (Quintelas, Sousa et al. 2006, Quintelas, Fernandes et al. 2008, Ding, Peng et al. 2013). We also tested pyrolysed biofilm samples (without Pd) and found negligible Cr(VI) reduction activity, which strongly suggests that palladium nano-crystal is the active catalytic component that performed Cr(VI) reduction in the biofilm-templated C-Pd nanocomposite catalyst.

Although we have shown that C-Pd nanocomposite is an effective catalyst for Cr(VI) reduction, it is also important to investigate its robustness. To this end, we examined the Cr(VI) reduction ability of various catalysts over 5 catalytic reactions (Figure 6.4).

Figure 6.4. Representative Cr(VI) reduction of C-Pd nanocomposite and biofilm-nanoPd (c-di-GMP) samples over 5 catalytic reactions. Initial concentration of 0.1 mM Cr(VI) and formic acid (1%) was introduced into the system every 10 minutes.

Surprisingly, we did not observe a significant drop in catalytic activity in C-Pd nanocomposite on the 5th catalytic reaction. C-Pd nanocomposite retained 93.6% of its initial Cr(VI) reduction capability while biofilm-nanoPd retained only about 46.2% of its initial Cr(VI) reduction capability after 5 catalytic reactions. The significant drop in catalytic activity of the biofilm-nanoPd (c-di-GMP) might be due to the cell

detachments and partial biofilm dispersal throughout the reaction process. On the other hand, the catalytic activity of C-Pd nanocomposite remained relatively constant over 5 catalytic reactions, which led us to believe that there is no significant leaching of palladium from the C-Pd nanocomposite during the reaction process. For biofilm-only samples, we did not observe much catalytic activity in the 5th catalytic cycle, possibly due to the exhaustion of adsorption site for Cr(VI) within the biofilm and loss of viability of the *S. oneidensis* bacteria to perform bioreduction of Cr(VI).

The high performance of C-Pd nanocomposite may be contributed by various factors, such as higher surface to volume ratio and porous structure of the nanocomposite to increase the availability of catalytic sites. One interesting observation is that sulfur, unlike nitrogen and phosphorus, is below the detection limit when we analysed the pyrolysed C-Pd nanocomposite sample using EDX. The possible removal of sulfur, a known palladium catalyst deactivator or poison (Albers, Pietsch et al. 2001), during pyrolysis might be beneficial to the performance of the C-Pd nanocomposite.

The formation of biotemplated catalyst via pyrolysis has been studied previously. Biofilm was used as a template to form catalytic mesoporous Pd electrodes via complete pyrolysis, where the biotemplated electrode is able to achieve a higher stable current density compared to electrochemically plated control electrode (Yates, Cusick et al. 2014). Adapting the idea, we chose to deploy a controlled pyrolysis so that we can retain some heteroatom-doped carbon that is attached to the palladium nanoparticles. Besides the fact that partial pyrolysis uses much lower energy as compared to complete pyrolysis, heteroatom-doped carbon may be catalytically active on specific reactions that can be useful in other applications. Many studies have looked at the catalytic effect of heteroatom doped carbon, especially its electrocatalytic

activity in oxygen reduction reaction and applications in energy and storage (Choi, Park et al. 2011, Yang, Jiang et al. 2011, Paraknowitsch and Thomas 2013, Zhu, Yin et al. 2013). Other studies have shown that metal catalyst that are supported on heteroatom-doped carbon have enhanced catalytic activity (Wu, Swaidan et al. 2008, Imran Jafri, Rajalakshmi et al. 2010, Zhou, Neyerlin et al. 2010). Although, in this study, pyrolyzed biofilm (heteroatom-doped carbon) without palladium showed negligible Cr(VI) reduction, it is unknown if the heteroatoms (P and N) enhanced the catalytic effect of C-Pd nanocomposite.

To this end, we tested the Cr(VI) reduction activity of C-Pd nanocomposite against commercial Pd nanopowder of similar particle size (<25nm) and Pd loading (~ 1 mg). Surprisingly, we found that the C-Pd nanocomposite exhibit 17% higher Cr(VI) reduction activity as compared to the commercial Pd nanopowder after 10 mins of reaction. Catalytic activity of commercial Pd nanopowder over multiple catalytic cycles was tested but it is not feasible to reuse the Pd nanopowder without loss of substantial amount Pd nanopowder during transferring of samples and that the catalyst recovery process used high energy intensive methods. We are currently unsure how the heteroatom-doped carbon support enhances the Pd nanocrystals' Cr(VI) reduction activity in our C-Pd nanocomposite catalytic system. Previous studies have attributed the catalytic enhancement of heteroatom-doped carbon support to increased catalyst particle dispersion (larger active sites), modified electronic structure of the catalyst (improved intrinsic catalytic activity) and better support/catalyst chemical bonding (improved durability) (Zhou, Neyerlin et al. 2010). This is an interesting question that is beyond the scope of this study, and warrants further investigation.

6.3.3. Environmental applications

In this study, we showed that MR-1 biofilms reduced Pd(II) to form Pd(0) nanocrystals (~10-20 nm) that were immobilised in the biofilm matrix and in cell membrane even under room temperature and bulk aerobic conditions. However, the introduction of Pd(II) increases the rate of cell detachment from the biofilm which posed a risk of biofilm dispersal and leeching of Pd. To create a more robust catalyst, we proceeded with controlled pyrolysis which leads to the carbonisation of biofilm and formation of abiotic heteroatom (N and P)-doped C-Pd nanocomposite catalyst. We assessed the catalytic capability of the pyrolysed biofilm-templated C-Pd catalyst which showed high Cr(VI) reduction, and maintained high reduction rate even on the 5th catalyst cycle, with possibly little or negligible amount of palladium leeching. Compared to the initial biofilm-Pd catalyst, the pyrolysed C-Pd nanocomposite does not have the problem of cell detachment due to high cytotoxicity of the reactants, nutrient requirement or uncontrollable biofilm growth and/or dispersal. The reusability of the pyrolysed heterogeneous catalyst greatly boosts the feasibility to deploy it in a fed-batch or continuous mode for the treatment of Cr(VI) contaminated water. We also showed that although the heteroatom-doped carbon support on its own have no Cr(VI) reduction activity, it may enhance the catalytic activity of the Pd nanocrystals in the C-Pd nanocomposite. Together, our results demonstrated an interdisciplinary and novel strategy for engineering biofilms towards the development of heteroatom-doped C-Pd nanocomposite catalyst that is applicable in Cr(VI) remediation. Using our study as a proof of concept, we propose that it may be possible to use a combination of organisms and reactive nano-metals to achieve desirable reactions for environmental remediation beyond Cr(VI) reduction, and possibly even fine chemical synthesis.

6.3.4. Conclusion

In this study, we propose an interdisciplinary and novel strategy towards the development of biofilm-templated heteroatom-doped catalytic system through bio-reduction and biofilm matrix-facilitated immobilisation of the *in situ* formed catalytic nanoparticles followed by controlled pyrolysis. Using *S. oneidensis* MR-1 as a model organism and palladium as a model precious metal, we showed that controlled pyrolysis leads to carbonisation of biofilm and formation of stable, abiotic heteroatom (N and P)-doped C-Pd nanocomposite catalyst. Moreover, the pyrolysed biofilm-templated C-Pd nanocomposite catalyst showed high Cr(VI) reduction, and maintained high reduction rate even on the 5th catalyst cycle. Although the heteroatom-doped carbon support on its own have no Cr(VI) reduction activity, it enhances the catalytic activity of the Pd nanocrystals in the C-Pd nanocomposite. Our results demonstrated an interdisciplinary and novel strategy for engineering biofilms towards the development of nanocomposite catalyst that is applicable in Cr(VI) reduction and other environmental remediation processes.

CHAPTER 7: CONCLUSIONS AND FUTURE RECOMMENDATIONS

7.1. Summary and Conclusions

The study aims to understand the mechanism of microbial synthesis of metal nanoparticles and develop biofilm-based nanocatalyst for multidimensional catalysis. Biofilm-based nanocatalysts were engineered via genetic manipulation and controlled pyrolysis to enhance robustness and reusability, for applications on environmental remediation such as TCE dechlorination and hexavalent chromium reduction.

In synthesis of metal nanomaterials by *S. oneidensis* MR-1, our result (Chapter 3 and 4) shows that outer membrane c-type cytochromes influence the size and activity of silver and silver sulfide NPs while [NiFe]-hydrogenases contribute to the synthesis of Pd NPs (Ng, Cai Tan et al. 2013). Moreover, we also reveal that in extracellular biosynthesis of NPs the usually neglected non-cell-associated NPs could have high catalytic activity, highlighting the need of novel methods, such as the use of biofilm that can efficiently retain extracellular NPs in the biosynthesis processes (Ng, Sivakumar et al. 2013).

The biofilm-mediated in situ synthesis and immobilisation of Pd NPs was achieved under bulk aerobic conditions, and the resultant nanocatalytic biofilm exhibit catalytic activity (Chapter 5). However, Pd(II) exposure inhibits cellular respiration and energy metabolism of *S. oneidensis* cells, posing a risk of biofilm detachment and decreased catalytic activity over time. The nanocatalytic biofilm was engineered to enhance its robustness and reusability via two approaches – genetic manipulation and physical alteration.

In previous studies, c-di-GMP has been reported to be able to influence the production of EPS and influence biofilm processes such as cell dispersal. In our study (Chapter 5), we showed that the insertion of plasmid *pYedQ2* can increase the production of cytoplasmic c-di-GMP in *S. oneidensis* MR-1, and this resulted in an increase in production of EPS and alleviate the problem of the biofilm detachment during the biofilm's exposure to Pd(II).

Further, we also showed that controlled pyrolysis leads to carbonisation of biofilm and formation of stable, abiotic biofilm-templated heteroatom (N and P)-doped C-Pd nanocomposite catalyst (Chapter 6). Moreover, the pyrolysed biofilm-templated C-Pd nanocomposite catalyst exhibit high Cr(VI) reduction activity, and maintained high reduction rate even on the 5th catalyst cycle. Although the heteroatom-doped carbon support on its own have no Cr(VI) reduction activity, it seems to enhance the catalytic activity of the Pd nanoparticles in the C-Pd nanocomposite.

While both genetic manipulation and physical alteration of the nanocatalytic biofilm produces promising results, there are various advantages and disadvantages of these approach as shown in Table 7.1.

Table 7.1. Comparison of genetic manipulation and physical alteration approach on performance of nanocatalytic biofilm.

	Genetic manipulation via c-di-GMP pathway	Physical alteration via controlled pyrolysis
Catalytic activity against nanocatalytic biofilm (wt)	No significant difference	Significantly higher

Production cost against nanocatalytic biofilm (wt)	No significant difference	Significantly higher
Catalyst recyclability	Low recyclability	High recyclability
Retain innate biofilm bioremediation process	Yes	No
Adsorption of contaminants	EPS and cell biomass can be an effective adsorbent.	Poor adsorbent
Scalability	Easily scalable	Poor scalability

Comparing with existing chemical and physical synthesis methods, there is a lack of precise control of the size and shape of nanoparticles using biofilm-based synthesis. However, as demonstrated, it is possible to influence the size and activity of the nanoparticles via genetic manipulation of the extracellular electron transport pathway of the biofilm-based synthesis of nanoparticle. One of the critical issues to be addressed is the scalability of this technology. While the use of genetic manipulation on the biofilm cells can be easily scalable, the same cannot be said for physical alteration due to technical difficulty of large-scale controlled pyrolysis. Hence, additional investigation, such as a pilot study, is needed to further this technology towards potential application.

This study adds on to the existing knowledge about the mechanistic details of microbial synthesis of metal nanomaterials. It also demonstrates that biofilm can be used to

synthesize Pd NPs, and the resultant nanocatalytic biofilm can be engineered to be more robust and feasible for large-scale industrial applications.

7.2. Future recommendation

The findings of this thesis work are a proof of concept where biofilms can be functionalised by *in situ* synthesized and immobilised Pd nanocatalyst within the EPS, and further engineered to enhance its robustness and reusability via genetic manipulations and physical alterations. Future recommendations involve integration of biofilm-based nanocatalyst with membrane technology and the exploration of biofilm-based nanocatalyst in other applications such as energy production.

7.2.1. Integration of biofilm-based nanocatalyst with membrane technology for environmental applications

Biofilm-based nanocatalysts that we have developed have shown to be able to remediate water micro-contaminants such as TCE and Cr(VI). Another important micro-contaminant is endocrine disrupting chemicals, such as 17 α -ethynylestradiol (EE2). EE2, a synthetic hormone, have received much attention due to its widespread occurrence and adverse effects on aquatic animals (Colborn, vom Saal et al. 1993). The removal of EE2 from water has been very challenging mostly because of its low concentration in environmental waters (Lange, Paull et al. 2009, Flores-Valverde, Horwood et al. 2010). Membrane technologies such as reverse osmosis and nanofiltration can remove EE2, but are energy and cost intensive. We have developed a relatively inexpensive and novel approach for concentrating EE2 through adsorption and elution using microporous polyethersulfone (PES) hollow fiber membrane in a cross-flow filtration system, which can be easily scalable for practical applications (Ng, Bope et al. 2017).

Previous studies have showed that estrogens can be degraded using oxidizing agent peroxymonosulfate (PMS), and the reaction can be catalysed by a PMS activator (Brienza, Ahmed et al. 2014). By coupling our biofilm-based nanocatalysts with microporous PES hollow fiber membrane system, our preliminary data shows that our biofilm-templated C-Pd nanocomposite, but not biotic nanocatalytic biofilm, is able to activate PMS to degrade the concentrated EE2 obtained from our adsorption-based filtration system. PMS is relatively unreactive until it is activated, in this case by the C-Pd nanocomposite, to produce strong oxidising agents such as reactive oxygen species (ROS) and radicals. The EPS component of the nanocatalytic biofilm contains many organic compounds which may quench the ROS and radicals. The optimisation of such coupling of the biofilm-based nanocatalyst and the microporous PES hollow fiber membrane system warrants further investigation (Figure 7.1).

Figure 7.1. Schematic diagram of the coupling of biofilm-templated C-Pd nanocomposite catalyst with microporous PES hollow fiber membrane system.

The use of biofilm-based nanocatalyst may be further integrated with other type of membrane system. Membranes with large pore sizes may be coated with reactive nanocatalytic biofilm for continuous reactions. Inert membranes, such as those made of ceramic, can even act as support for nanocatalytic biofilm and undergo controlled pyrolysis as an approach to functionalise the membrane with catalytic biofilm-templated C-Pd nanocomposite. Such processes need to be optimised to be feasible for large-scale industrial processes.

7.2.2. Biofilm-based nanocatalyst for energy-related applications

Current research on microbial synthesis of nanoparticles focus on environmental remediation applications, such as TCE dechlorination (Chapter 5) and hexavalent chromium reduction (Chapter 6). However, little emphasis is placed on other exotic applications such as energy production and storage. Besides silver and Pd, the biofilm-based synthesis of metal nanoparticles can theoretically be extended to many other metal or metalloid materials. This creates new opportunities for novel energy-related applications involving the use of highly catalytic nanoparticles.

Up to date, microbial fuel cells have been shown to produce energy by harnessing the metabolic energy of biofilms (Liu, Ramnarayanan et al. 2004), but little is known about how functionalising biofilm with reactive nanoparticles can aid electron transfer between the biofilm and the electrode. Conductive metal(loid) nanoparticles that are synthesized and immobilised within the EPS may reduce the overall electrical impedance of the biofilm and aid the extracellular electron transfer between the biofilm and the electrode. The heteroatoms (N and P) within the biofilm-templated C-Pd

nanocomposite may also improve the conductivity and catalytic activity of the catalyst which require further extensive studies.

REFERENCES

Adav, S. S. and D.-J. Lee (2008). "Extraction of extracellular polymeric substances from aerobic granule with compact interior structure." Journal of hazardous materials **154(1)**: 1120-1126.

Albers, P., J. Pietsch and S. F. Parker (2001). "Poisoning and deactivation of palladium catalysts." Journal of Molecular Catalysis A: Chemical **173(1-2)**: 275-286.

Ali, I. (2012). "New Generation Adsorbents for Water Treatment." Chemical Reviews **112(10)**: 5073-5091.

Arciola, C. R., D. Campoccia, P. Speziale, L. Montanaro and J. W. Costerton (2012). "Biofilm formation in Staphylococcus implant infections. A review of molecular mechanisms and implications for biofilm-resistant materials." Biomaterials **33(26)**: 5967-5982.

Aris, A. Z., A. S. Shamsuddin and S. M. Praveena (2014). "Occurrence of 17 α -ethynylestradiol (EE2) in the environment and effect on exposed biota: a review." Environment International **69**: 104-119.

Atkins, P. (2010). Shriver and Atkins' inorganic chemistry, Oxford University Press, USA.

Bai, H.-J., B.-S. Yang, C.-J. Chai, G.-E. Yang, W.-L. Jia and Z.-B. Yi (2011). "Green synthesis of silver nanoparticles using *Rhodobacter Sphaeroides*." World Journal of Microbiology and Biotechnology **27(11)**: 2723.

Bansal, R. C. and M. Goyal (2005). Activated carbon adsorption, CRC press.

Barnhart, J. (1997). "Occurrences, Uses, and Properties of Chromium." Regulatory Toxicology and Pharmacology **26(1)**: S3-S7.

Baruah, D., R. N. Das, S. Hazarika and D. Konwar (2015). "Biogenic synthesis of cellulose supported Pd(0) nanoparticles using hearth wood extract of *Artocarpus lakoocha* Roxb — A green, efficient and versatile catalyst for Suzuki and Heck coupling in water under microwave heating." Catalysis Communications **72**: 73-80.

Beauregard, D. A., P. Yong, L. E. Macaskie and M. L. Johns (2010). "Using non-invasive magnetic resonance imaging (MRI) to assess the reduction of Cr(VI) using a biofilm–palladium catalyst." Biotechnology and Bioengineering **107(1)**: 11-20.

Belchik, S. M., D. W. Kennedy, A. C. Dohnalkova, Y. Wang, P. C. Sevinc, H. Wu, Y. Lin, H. P. Lu, J. K. Fredrickson and L. Shi (2011). "Extracellular Reduction of Hexavalent Chromium by Cytochromes MtrC and OmcA of *Shewanella oneidensis* MR-1." Applied and Environmental Microbiology **77(12)**: 4035-4041.

Bellin, D. L., H. Sakhtah, J. K. Rosenstein, P. M. Levine, J. Thimot, K. Emmett, L. E. P. Dietrich and K. L. Shepard (2014). "Integrated circuit-based electrochemical sensor for spatially resolved detection of redox-active metabolites in biofilms." Nature Communications **5**: 3256.

Bennett, J. A., I. P. Mikheenko, K. Deplanche, I. J. Shannon, J. Wood and L. E. Macaskie (2013). "Nanoparticles of palladium supported on bacterial biomass: New re-usable heterogeneous catalyst with comparable activity to homogeneous colloidal Pd in the Heck reaction." Applied Catalysis B: Environmental **140–141(0)**: 700-707.

Berendsen, H. J. C., J. P. M. Postma, W. F. Van Gunsteren, A. DiNola and J. R. Haak (1984). "Molecular dynamics with coupling to an external bath." J. Chem. Phys **81**: 3684-3690.

Berendsen, H. J. C., J. P. M. Postma, W. F. van Gunsteren and H. J. (1981). "Interaction models for water in relation to protein hydration. ." In Intermolecular Forces, B. Pullman (ed), Dordrecht, The Netherlands: 331-332.

Bigall, N. C., M. Reitzig, W. Naumann, P. Simon, K.-H. van Pée and A. Eychmüller (2008). "Fungal Templates for Noble-Metal Nanoparticles and Their Application in Catalysis." Angewandte Chemie International Edition **47(41)**: 7876-7879.

Bishop, P. L., T. C. Zhang and Y.-C. Fu (1995). "Effects of biofilm structure, microbial distributions and mass transport on biodegradation processes." Water Science and Technology **31(1)**: 143-152.

Blowes, D. W., C. J. Ptacek and J. L. Jambor (1997). "In-Situ Remediation of Cr(VI)-Contaminated Groundwater Using Permeable Reactive Walls: Laboratory Studies." Environmental Science & Technology **31(12)**: 3348-3357.

Bornscheuer, U., G. Huisman, R. Kazlauskas, S. Lutz, J. Moore and K. Robins (2012). "Engineering the third wave of biocatalysis." Nature **485**: 185-194.

Bosire, E. M., L. M. Blank and M. A. Rosenbaum (2016). "Strain and substrate dependent redox mediator and electricity production by *Pseudomonas aeruginosa*." Applied and Environmental Microbiology.

Branda, S. S., Å. Vik, L. Friedman and R. Kolter (2005). "Biofilms: the matrix revisited." Trends in microbiology **13(1)**: 20-26.

Brienza, M., M. M. Ahmed, A. Escande, G. Plantard, L. Scrano, S. Chiron, S. Bufo and V. Goetz (2014). "Relevance of a photo-Fenton like technology based on peroxymonosulphate for 17 β -estradiol removal from wastewater." Chemical Engineering Journal **257**: 191-199.

Broberg, C. A., T. J. Calder and K. Orth (2011). "Vibrio parahaemolyticus cell biology and pathogenicity determinants." Microbes and Infection **13(12–13)**: 992-1001.

Bücking, C., A. Piepenbrock, A. Kappler and J. Gescher (2012). "Outer-membrane cytochrome-independent reduction of extracellular electron acceptors in *Shewanella oneidensis*." Microbiology **158(Pt 8)**: 2144-2157.

Bunge, M., L. S. Søbjerg, A.-E. Rotaru, D. Gauthier, A. T. Lindhardt, G. Hause, K. Finster, P. Kingshott, T. Skrydstrup and R. L. Meyer (2010). "Formation of palladium(0) nanoparticles at microbial surfaces." Biotechnology and Bioengineering **107(2)**: 206-215.

Burmølle, M., D. Ren, T. Bjarnsholt and S. J. Sørensen (2014). "Interactions in multispecies biofilms: do they actually matter?" Trends in microbiology.

Bussi, G., D. Donadio and M. Parrinello (2007). "Canonical sampling through velocity rescaling." J. Chem. Phys **126**: 014101-014107.

Cao, B., B. Ahmed, D. W. Kennedy, Z. Wang, L. Shi, M. J. Marshall, J. K. Fredrickson, N. G. Isern, P. D. Majors and H. Beyenal (2011). "Contribution of

extracellular polymeric substances from *Shewanella* sp. HRCR-1 biofilms to U(VI) immobilization." Environmental Science and Technology **45(13)**: 5483-5490.

Cao, B., B. Ahmed, D. W. Kennedy, Z. Wang, L. Shi, M. J. Marshall, J. K. Fredrickson, N. G. Isern, P. D. Majors and H. Beyenal (2011). "Contribution of extracellular polymeric substances from *Shewanella* sp. HRCR-1 biofilms to U(VI) immobilization." Environmental Science & Technology **45(13)**: 5483-5490.

Cao, B., L. Shi, R. N. Brown, Y. Xiong, J. K. Fredrickson, M. F. Romine, M. J. Marshall, M. S. Lipton and H. Beyenal (2011). "Extracellular polymeric substances from *Shewanella* sp. HRCR-1 biofilms: characterization by infrared spectroscopy and proteomics." Environmental Microbiology **13(4)**: 1018-1031.

Cao, B., L. Shi, R. N. Brown, Y. Xiong, J. K. Fredrickson, M. F. Romine, M. J. Marshall, M. S. Lipton and H. Beyenal (2011). "Extracellular polymeric substances from *Shewanella* sp. HRCR-1 biofilms: characterization by infrared spectroscopy and proteomics." Environmental Microbiology **13(4)**: 1018-1031.

Cao, B., L. Shi, R. N. Brown, Y. J. Xiong, J. K. Fredrickson, M. F. Romine, M. J. Marshall, M. S. Lipton and H. Beyenal (2011). "Extracellular polymeric substances from *Shewanella* sp HRCR-1 biofilms: characterization by infrared spectroscopy and proteomics." Environmental Microbiology **13(4)**: 1018-1031.

Cartinella, J. L., T. Y. Cath, M. T. Flynn, G. C. Miller, K. W. Hunter and A. E. Childress (2006). "Removal of Natural Steroid Hormones from Wastewater Using Membrane Contactor Processes." Environmental Science & Technology **40(23)**: 7381-7386.

Carvalho, R., L. Ceriani, A. Ippolito and T. Lettieri (2015). "Development of the First Watch List under the Environmental Quality Standards Directive." European Commission Joint Research Center, Ispra, Italy.

Chai, L., S. Huang, Z. Yang, B. Peng, Y. Huang and Y. Chen (2009). "Cr (VI) remediation by indigenous bacteria in soils contaminated by chromium-containing slag." Journal of Hazardous Materials **167(1–3)**: 516-522.

Chao, L., S. Rakshe, M. Leff and A. M. Spormann (2013). "PdeB, a Cyclic Di-GMP-Specific Phosphodiesterase That Regulates *Shewanella oneidensis* MR-1 Motility and Biofilm Formation." Journal of Bacteriology **195(17)**: 3827-3833.

Chebeir, M. and H. Liu (2016). "Kinetics and Mechanisms of Cr(VI) Formation via the Oxidation of Cr(III) Solid Phases by Chlorine in Drinking Water." Environmental Science & Technology **50(2)**: 701-710.

Chen, C.-Y., G. Nace and P. Irwin (2003). "A 6x6 drop plate method for simultaneous colony counting and MPN enumeration of *Campylobacter jejuni*, *Listeria monocytogenes*, and *Escherichia coli*." Journal of Microbiological Methods **55**: 475-479.

Chidambaram, D., T. Hennebel, S. Taghavi, J. Mast, N. Boon, W. Verstraete, D. van der Lelie and J. P. Fitts (2010). "Concomitant Microbial Generation of Palladium Nanoparticles and Hydrogen To Immobilize Chromate." Environmental Science & Technology **44(19)**: 7635-7640.

Choi, C. H., S. H. Park and S. I. Woo (2011). "Heteroatom doped carbons prepared by the pyrolysis of bio-derived amino acids as highly active catalysts for oxygen electro-reduction reactions." Green Chemistry **13(2)**: 406-412.

Choi, O. and Z. Hu (2008). "Size Dependent and Reactive Oxygen Species Related Nanosilver Toxicity to Nitrifying Bacteria." Environmental Science & Technology **42(12)**: 4583-4588.

Coe, T. S., P. B. Hamilton, D. Hodgson, G. C. Paull, J. R. Stevens, K. Sumner and C. R. Tyler (2008). "An Environmental Estrogen Alters Reproductive Hierarchies, Disrupting Sexual Selection in Group-Spawning Fish." Environmental Science & Technology **42(13)**: 5020-5025.

Cohen, Y. (2001). "Biofiltration - the treatment of fluids by microorganisms immobilized into the filter bedding material: a review." Bioresource technology **77(3)**: 257-274.

Colborn, T., F. S. vom Saal and A. M. Soto (1993). "Developmental effects of endocrine-disrupting chemicals in wildlife and humans." Environmental health perspectives **101(5)**: 378.

Coleman, H. M., E. J. Routledge, J. P. Sumpter, B. R. Egdins and J. A. Byrne (2004). "Rapid loss of estrogenicity of steroid estrogens by UVA photolysis and photocatalysis over an immobilised titanium dioxide catalyst." Water Research **38(14–15)**: 3233-3240.

Comerton, A. M., R. C. Andrews, D. M. Bagley and C. Hao (2008). "The rejection of endocrine disrupting and pharmaceutically active compounds by NF and RO membranes as a function of compound and water matrix properties." Journal of Membrane Science **313(1–2)**: 323-335.

Comte, S., G. Guibaud and M. Baudu (2006). "Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and EPS

complexation properties: Part I. Comparison of the efficiency of eight EPS extraction methods." Enzyme and Microbial Technology **38(1)**: 237-245.

Corbos, E. C., P. R. Ellis, J. Cookson, V. Briois, T. I. Hyde, G. Sankar and P. T. Bishop (2013). "Tuning the properties of PdAu bimetallic nanocatalysts for selective hydrogenation reactions." Catalysis Science & Technology **3(11)**: 2934-2943.

Costa, M. (2003). "Potential hazards of hexavalent chromate in our drinking water." Toxicology and Applied Pharmacology **188(1)**: 1-5.

Costa, M. and C. B. Klein (2006). "Toxicity and carcinogenicity of chromium compounds in humans." Critical reviews in toxicology **36(2)**: 155-163.

Dahl, J. A., B. L. S. Maddux and J. E. Hutchison (2007). "Toward Greener Nanosynthesis." Chemical Reviews **107(6)**: 2228-2269.

Dangcong, P., N. Bernet, J.-P. Delgenes and R. Moletta (1999). "Aerobic granular sludge—a case report." Water Research **33(3)**: 890-893.

Darden, T., D. York and L. Pedersen (1993). "Particle mesh Ewald: An $N \log(N)$ method for Ewald sums." J. Chem. Phys **98**: 10089-10092.

Das, N. (2010). "Recovery of precious metals through biosorption - A review." Hydrometallurgy **103(1-4)**: 180-189.

De Corte, S., S. Bechstein, A. R. Lokanathan, J. Kijms, N. Boon and R. L. Meyer (2012). "Comparison of bacterial cells and amine-functionalized abiotic surfaces as support for Pd nanoparticle synthesis." Colloids and Surfaces B: Biointerfaces **102**: 898-904.

De Corte, S., T. Hennebel, B. De Gusseme, W. Verstraete and N. Boon (2012).

"Bio-palladium: From metal recovery to catalytic applications." Microbial

Biotechnology **5(1)**: 5-17.

De Corte, S., T. Hennebel, J. P. Fitts, T. Sabbe, V. Bliznuk, S. Verschuere, D.

Van Der Lelie, W. Verstraete and N. Boon (2011). "Biosupported bimetallic Pd-Au

nanocatalysts for dechlorination of environmental contaminants." Environmental

Science and Technology **45(19)**: 8506-8513.

De Corte, S., T. Hennebel, S. Verschuere, C. Cuvelier, W. Verstraete and N.

Boon (2011). "Gold nanoparticle formation using *Shewanella oneidensis*: A fast

biosorption and slow reduction process." Journal of Chemical Technology and

Biotechnology **86(4)**: 547-553.

De Corte, S., T. Sabbe, T. Hennebel, L. Vanhaecke, B. De Gusseme, W.

Verstraete and N. Boon (2012). "Doping of biogenic Pd catalysts with Au enables

dechlorination of diclofenac at environmental conditions." Water Research **46(8)**:

2718-2726.

De Gusseme, B., T. Hennebel, E. Christiaens, H. Saveyn, K. Verbeken, J. P.

Fitts, N. Boon and W. Verstraete (2011). "Virus disinfection in water by biogenic

silver immobilized in polyvinylidene fluoride membranes." Water Research **45(4)**:

1856-1864.

De Windt, W., N. Boon, J. Van den Bulcke, L. Rubberecht, F. Prata, J. Mast, T.

Hennebel and W. Verstraete (2006). "Biological control of the size and reactivity of

catalytic Pd(0) produced by *Shewanella oneidensis*." Antonie van Leeuwenhoek,

International Journal of General and Molecular Microbiology **90(4)**: 377-389.

Deplanche, K., I. Caldelari, I. P. Mikheenko, F. Sargent and L. E. Macaskie

(2010). "Involvement of hydrogenases in the formation of highly catalytic Pd(0) nanoparticles by bioreduction of Pd(II) using *Escherichia coli* mutant strains."

Microbiology **156(9)**: 2630-2640.

Deplanche, K., M. L. Merroun, M. Casadesus, D. T. Tran, I. P. Mikheenko, J. A.

Bennett, J. Zhu, I. P. Jones, G. A. Attard, J. Wood, S. Selenska-Pobell and L. E.

Macaskie (2012). "Microbial synthesis of core/shell gold/palladium nanoparticles for applications in green chemistry." Journal of the Royal Society Interface **9(72)**: 1705-1712.

Ding, Y., N. Peng, Y. Du, L. Ji and B. Cao (2013). "Disruption of Putrescine

Biosynthesis in *Shewanella oneidensis* Enhances Biofilm Cohesiveness and

Performance in Cr(VI) Immobilization." Applied and Environmental Microbiology.

Durán, N., P. D. Marcato, M. Durán, A. Yadav, A. Gade and M. Rai (2011).

"Mechanistic aspects in the biogenic synthesis of extracellular metal nanoparticles by peptides, bacteria, fungi, and plants." Applied Microbiology and Biotechnology **90(5)**: 1609-1624.

Durán, N. and A. Seabra (2012). "Metallic oxide nanoparticles: state of the art in

biogenic syntheses and their mechanisms." Applied Microbiology and Biotechnology **95(2)**: 275-288.

El-Naggar, M. Y., G. Wanger, K. M. Leung, T. D. Yuzvinsky, G. Southam, J.

Yang, W. M. Lau, K. H. Nealson and Y. A. Gorby (2010). "Electrical transport along bacterial nanowires from *Shewanella oneidensis* MR-1." Proceedings of the National Academy of Sciences **107(42)**: 18127-18131.

Essmann, U., L. Perera, M. L. Berkowitz, T. Darden, H. Lee and L. Pedersen

(1995). "A smooth particle mesh Ewald method." *J. Chem. Phys* 103: 8577-8593.

Flemming, H.-C. (2011). "The perfect slime." *Colloids and Surfaces B: Biointerfaces*

86(2): 251-259.

Flemming, H.-C. and J. Wingender (2010). "The biofilm matrix." *Nature Reviews*

Microbiology **8(9):** 623-633.

Flemming, H. (2011). "The perfect slime." *Colloids and Surfaces B: Biointerfaces*

86: 251-259.

Flemming, H. and J. Wingender (2010). "The biofilm matrix." *Nature Reviews in*

Microbiology **8:** 623-633.

Flores-Valverde, A. M., J. Horwood and E. M. Hill (2010). "Disruption of the

Steroid Metabolome in Fish Caused by Exposure to the Environmental Estrogen

17 α -Ethinylestradiol." *Environmental Science & Technology* **44(9):** 3552-3558.

Forrez, I., M. Carballa, G. Fink, A. Wick, T. Hennebel, L. Vanhaecke, T. Ternes,

N. Boon and W. Verstraete (2011). "Biogenic metals for the oxidative and reductive

removal of pharmaceuticals, biocides and iodinated contrast media in a polishing

membrane bioreactor." *Water Research* **45(4):** 1763-1773.

Fredrickson, J. K., M. F. Romine, A. S. Beliaev, J. M. Auchtung, M. E. Driscoll,

T. S. Gardner, K. H. Nealson, A. L. Osterman, G. Pinchuk, J. L. Reed, D. A.

Rodionov, J. L. M. Rodrigues, D. A. Saffarini, M. H. Serres, A. M. Spormann, I.

B. Zhulin and J. M. Tiedje (2008). "Towards environmental systems biology of

Shewanella." *Nature Reviews Microbiology* **6(8):** 592-603.

Fu, H., R. P. S. Suri, R. F. Chimchirian, E. Helmig and R. Constable (2007).

"Ultrasound-Induced Destruction of Low Levels of Estrogen Hormones in Aqueous Solutions." Environmental Science & Technology **41(16)**: 5869-5874.

Gadd, G. M. (2010). "Metals, minerals and microbes: geomicrobiology and bioremediation." Microbiology **156(3)**: 609-643.

Gaulke, L. S., S. E. Strand, T. F. Kalhorn and H. D. Stensel (2009). "Estrogen Biodegradation Kinetics and Estrogenic Activity Reduction for Two Biological Wastewater Treatment Methods." Environmental Science & Technology **43(18)**: 7111-7116.

Gauthier, D., L. S. Søbjerger, K. M. Jensen, A. T. Lindhardt, M. Bunge, K. Finster, R. L. Meyer and T. Skrydstrup (2010). "Environmentally benign recovery and reactivation of palladium from industrial waste by using gram - negative bacteria." ChemSusChem **3(9)**: 1036-1039.

Gjermansen, M., P. Ragas and T. Tolker-Nielsen (2006). "Proteins with GGDEF and EAL domains regulate *Pseudomonas putida* biofilm formation and dispersal." FEMS Microbiology Letters **265(2)**: 215-224.

Gorby, Y. A., S. Yanina, J. S. McLean, K. M. Rosso, D. Moyles, A. Dohnalkova, T. J. Beveridge, I. S. Chang, B. H. Kim, K. S. Kim, D. E. Culley, S. B. Reed, M. F. Romine, D. A. Saffarini, E. A. Hill, L. Shi, D. A. Elias, D. W. Kennedy, G. Pinchuk, K. Watanabe, S. i. Ishii, B. Logan, K. H. Nealson and J. K. Fredrickson (2006). "Electrically conductive bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms." Proceedings of the National Academy of Sciences **103(30)**: 11358-11363.

- Green, C., R. Williams, R. Kanda, J. Churchley, Y. He, S. Thomas, P. Goonan, A. Kumar and S. Jobling** (2013). "Modeling of Steroid Estrogen Contamination in UK and South Australian Rivers Predicts Modest Increases in Concentrations in the Future." Environmental Science & Technology **47(13)**: 7224-7232.
- Gross, R., B. Hauer, K. Otto and A. Schmid** (2007). "Microbial biofilms: New catalysts for maximizing productivity of long-term biotransformations." Biotechnology and Bioengineering **98(6)**: 1123-1134.
- Gross, R., K. Lang, K. Bühler and A. Schmid** (2010). "Characterization of a biofilm membrane reactor and its prospects for fine chemical synthesis." Biotechnology and Bioengineering **105(4)**: 705-717.
- Grover, D. P., J. L. Zhou, P. E. Frickers and J. W. Readman** (2011). "Improved removal of estrogenic and pharmaceutical compounds in sewage effluent by full scale granular activated carbon: Impact on receiving river water." Journal of Hazardous Materials **185(2-3)**: 1005-1011.
- Guha, H., K. Jayachandran and F. Maurrasse** (2001). "Kinetics of chromium (VI) reduction by a type strain *Shewanella* alga under different growth conditions." Environmental Pollution **115(2)**: 209-218.
- Habibul, N., Y. Hu, Y.-K. Wang, W. Chen, H.-Q. Yu and G.-P. Sheng** (2016). "Bioelectrochemical Chromium(VI) Removal in Plant-Microbial Fuel Cells." Environmental Science & Technology **50(7)**: 3882-3889.
- Halan, B., K. Buehler and A. Schmid** (2012). "Biofilms as living catalysts in continuous chemical syntheses." Trends in Biotechnology **30(9)**: 453-465.

Halan, B., A. Schmid and K. Buchler (2010). "Maximizing the Productivity of Catalytic Biofilms on Solid Supports in Membrane Aerated Reactors." Biotechnology and bioengineering **106(4)**: 516-527.

Hammer, B. K. and B. L. Bassler (2003). "Quorum sensing controls biofilm formation in *Vibrio cholerae*." Molecular Microbiology **50(1)**: 101-104.

Han, J., W. Qiu, Z. Cao, J. Hu and W. Gao (2013). "Adsorption of ethinylestradiol (EE2) on polyamide 612: Molecular modeling and effects of water chemistry." Water Research **47(7)**: 2273-2284.

Hanselman, T. A., D. A. Graetz and A. C. Wilkie (2003). "Manure-Borne Estrogens as Potential Environmental Contaminants: A Review." Environmental Science & Technology **37(24)**: 5471-5478.

Harris, H. W., M. Y. El-Naggar and K. H. Nealson (2012). "*Shewanella oneidensis* MR-1 chemotaxis proteins and electron-transport chain components essential for congregation near insoluble electron acceptors." Biochemical Society Transactions **40(6)**: 1167-1177.

He, S., Z. Guo, Y. Zhang, S. Zhang, J. Wang and N. Gu (2007). "Biosynthesis of gold nanoparticles using the bacteria *Rhodopseudomonas capsulata*." Materials Letters **61(18)**: 3984-3987.

Heidelberg, J. F., I. T. Paulsen, K. E. Nelson, E. J. Gaidos, W. C. Nelson, T. D. Read, J. A. Eisen, R. Seshadri, N. Ward and B. Methe (2002). "Genome sequence of the dissimilatory metal ion–reducing bacterium *Shewanella oneidensis*." Nature biotechnology **20(11)**: 1118-1123.

Hengge, R. (2009). "Principles of c-di-GMP signalling in bacteria." Nature Reviews Microbiology **7(4)**: 263-273.

Hennebel, T., S. De Corte, L. Vanhaecke, K. Vanherck, I. Forrez, B. De Gusseme, P. Verhagen, K. Verbeken, B. Van der Bruggen, I. Vankelecom, N. Boon and W. Verstraete (2010). "Removal of diatrizoate with catalytically active membranes incorporating microbially produced palladium nanoparticles." Water Research **44(5)**: 1498-1506.

Hennebel, T., S. De Corte, W. Verstraete and N. Boon (2012). "Microbial production and environmental applications of Pd nanoparticles for treatment of halogenated compounds." Current Opinion in Biotechnology.

Hennebel, T., B. De Gusseme, N. Boon and W. Verstraete (2009). "Biogenic metals in advanced water treatment." Trends in Biotechnology **27(2)**: 90-98.

Hennebel, T., H. Simoen, W. De Windt, M. Verloo, N. Boon and W. Verstraete (2009). "Biocatalytic dechlorination of trichloroethylene with bio-palladium in a pilot-scale membrane reactor." Biotechnology and Bioengineering **102(4)**: 995-1002.

Hennebel, T., S. Van Nevel, S. Verschuere, S. De Corte, B. De Gusseme, C. Cuvelier, J. P. Fitts, D. Van der Lelie, N. Boon and W. Verstraete (2011). "Palladium nanoparticles produced by fermentatively cultivated bacteria as catalyst for diatrizoate removal with biogenic hydrogen." Applied Microbiology & Biotechnology **91(5)**: 1435-1445.

Hentzer, M. and M. Givskov (2003). "Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections." The Journal of Clinical Investigation **112(9)**: 1300-1307.

Heo, J., L. K. Boateng, J. R. Flora, H. Lee, N. Her, Y.-G. Park and Y. Yoon (2013).

"Comparison of flux behavior and synthetic organic compound removal by forward osmosis and reverse osmosis membranes." Journal of membrane science **443**: 69-82.

Hernandez, M. E., A. Kappler and D. K. Newman (2004). "Phenazines and other redox-active antibiotics promote microbial mineral reduction." Applied and environmental microbiology **70(2)**: 921-928.

Hess, B., H. Bekker, H. J. C. Berendsen and J. G. E. M. Fraaije (1997). "LINCS: a Linear Constraint Solver for Molecular Simulation." J. Comput. Chem **18**: 1463-1472.

Heugebaert, T. S. A., S. De Corte, T. Sabbe, T. Hennebel, W. Verstraete, N. Boon and C. V. Stevens (2012). "Biodeposited Pd/Au bimetallic nanoparticles as novel Suzuki catalysts." Tetrahedron Letters **53(11)**: 1410-1412.

Holst, G., R. N. Glud, M. Kühl and I. Klimant (1997). "A microoptode array for fine-scale measurement of oxygen distribution." Sensors and Actuators B: Chemical **38(1-3)**: 122-129.

Hosseinkhani, B., T. Hennebel, S. Van Nevel, S. Verschuere, M. M. Yakimov, S. Cappello, M. Blaghen and N. Boon (2014). "Biogenic Nanopalladium Based Remediation of Chlorinated Hydrocarbons in Marine Environments." Environmental Science & Technology **48(1)**: 550-557.

Huang, Y., H. Ma, S. Wang, M. Shen, R. Guo, X. Cao, M. Zhu and X. Shi (2012). "Efficient Catalytic Reduction of Hexavalent Chromium Using Palladium Nanoparticle-Immobilized Electrospun Polymer Nanofibers." ACS Applied Materials & Interfaces **4(6)**: 3054-3061.

Hub, S. J., L. B. De Groot and D. Van der Spee (2010). "g_wham- A Free Weighted Histogram Analysis Implemented Including Robust Error and Autocorrelation Estimation." J. Chem. Theory Comput **6**: 3713-3720.

International Agency for Research on Cancer (1987). "Overall evaluations of carcinogenicity: an updating of IARC monographs" **Volumes 1 to 42**. Lyon: IARC.

Hung, Y.-T., H. H. Lo, L. K. Wang, J. R. Taricska and K. H. Li (2005). Granular activated carbon adsorption. Physicochemical treatment processes, Springer: 573-633.

Imran Jafri, R., N. Rajalakshmi and S. Ramaprabhu (2010). "Nitrogen doped graphene nanoplatelets as catalyst support for oxygen reduction reaction in proton exchange membrane fuel cell." Journal of Materials Chemistry **20(34)**: 7114-7117.

Irmak, S., O. Erbatur and A. Akgerman (2005). "Degradation of 17 β -estradiol and bisphenol A in aqueous medium by using ozone and ozone/UV techniques." Journal of Hazardous Materials **126(1–3)**: 54-62.

Jahn, A. and P. Nielsen (1995). "Extraction of extracellular polymeric substances (EPS) from biofilms using a cation exchange resin." Water Science and Technology **32(8)**: 157-164.

Jain, N., A. Bhargava, S. Majumdar, J. C. Tarafdar and J. Panwar (2011). "Extracellular biosynthesis and characterization of silver nanoparticles using *Aspergillus flavus* NJP08: A mechanism perspective." Nanoscale **3(2)**: 635-641.

Jenal, U. and J. Malone (2006). "Mechanisms of Cyclic-di-GMP Signaling in Bacteria." Annual Review of Genetics **40(1)**: 385-407.

Jiang, W., B. Kim, J. Rutka and W. Chan (2008). "Nanoparticle-mediated cellular response is size-dependent." Nature Nanotechnology **3**: 145-150.

Jiang, W., Y. S. Kim, J. T. Rutka and C. W. Chan (2008).

"Nanoparticle-mediated cellular response is size-dependent." Nat Nano **3(3)**: 145-150.

Johnson, A. C. (2010). "Natural Variations in Flow Are Critical in Determining Concentrations of Point Source Contaminants in Rivers: An Estrogen Example." Environmental Science & Technology **44(20)**: 7865-7870.

Jorgensen, W. L. and J. Tirado Rives (1988). "The OPLS [optimized potential for liquid simulations] potential functions for proteins, energy minimizations for crystals of cyclic peptides and crambin." J. Am. Chem. Soc **110**: 1657-1666.

Juibari, M. M., S. Abbasalizadeh, G. S. Jouzani and M. Noruzi (2011). "Intensified biosynthesis of silver nanoparticles using a native extremophilic *Ureibacillus thermosphaericus* strain." Materials Letters **65(6)**: 1014-1017.

Jung, J. H., T. J. Park, S. Y. Lee and T. S. Seo (2012). "Homogeneous Biogenic Paramagnetic Nanoparticle Synthesis Based on a Microfluidic Droplet Generator." Angewandte Chemie **124(23)**: 5732-5735.

K, S. K., A. R. P. Arumugam and S. Berchmans (2011). "Synthesis of Gold Nanoparticles: An Ecofriendly Approach Using *Hansenula anomala*." ACS Applied Materials & Interfaces **3(5)**: 1418-1425.

Kalathil, S., J. Lee and M. H. Cho (2011). "Electrochemically active biofilm-mediated synthesis of silver nanoparticles in water." Green Chemistry **13(6)**: 1482-1485.

Kalimuthu, K., R. Suresh Babu, D. Venkataraman, M. Bilal and S. Gurunathan (2008). "Biosynthesis of silver nanocrystals by *Bacillus licheniformis*." Colloids and Surfaces B: Biointerfaces **65(1)**: 150-153.

Kalishwaralal, K., V. Deepak, S. Ram Kumar Pandian, M. Kottaisamy, S. BarathManiKanth, B. Kartikeyan and S. Gurunathan (2010). "Biosynthesis of silver and gold nanoparticles using *Brevibacterium casei*." Colloids and Surfaces B: Biointerfaces **77(2)**: 257-262.

Katz, S. A. and H. Salem (1993). "The toxicology of chromium with respect to its chemical speciation: A review." Journal of Applied Toxicology **13(3)**: 217-224.

Kessler, B., V. Lorenzo and K. Timmis (1992). "A general system to integrate lacZ fusions into the chromosomes of gram-negative eubacteria: regulation of the P_m promoter of the TOL plasmid studied with all controlling elements in monocopy." Molecular and General Genetics MGG **233(1-2)**: 293-301.

Khan, M. M., S. A. Ansari, M. I. Amal, J. Lee and M. H. Cho (2013). "Highly visible light active Ag@TiO₂ nanocomposites synthesized using an electrochemically active biofilm: a novel biogenic approach." Nanoscale **5(10)**: 4427-4435.

Khan, M. M., J. Lee and M. H. Cho (2013). "Electrochemically active biofilm mediated bio-hydrogen production catalyzed by positively charged gold nanoparticles." International Journal of Hydrogen Energy **38(13)**: 5243-5250.

Kim, J.-H., D.-C. Choi, K.-M. Yeon, S.-R. Kim and C.-H. Lee (2011). "Enzyme-Immobilized Nanofiltration Membrane To Mitigate Biofouling Based on Quorum Quenching." Environmental Science & Technology **45(4)**: 1601-1607.

Kolter, R. and E. P. Greenberg (2006). "Microbial sciences: the superficial life of microbes." Nature **441(7091)**: 300-302.

Konishi, Y., T. Tsukiyama, T. Tachimi, N. Saitoh, T. Nomura and S. Nagamine (2007). "Microbial deposition of gold nanoparticles by the metal-reducing bacterium *Shewanella algae*." Electrochimica Acta **53(1)**: 186-192.

Krasteva, P. V., J. C. N. Fong, N. J. Shikuma, S. Beyhan, M. V. A. S. Navarro, F. H. Yildiz and H. Sondermann (2010). "Vibrio cholerae VpsT Regulates Matrix Production and Motility by Directly Sensing Cyclic di-GMP." Science **327(5967)**: 866-868.

Kryukov, A. I., A. L. Stroyuk, N. N. Zin'chuk, A. V. Korzhak and S. Y. Kuchmii (2004). "Optical and catalytic properties of Ag₂S nanoparticles." Journal of Molecular Catalysis A: Chemical **221(1-2)**: 209-221.

Kuchma, S. L., A. E. Ballok, J. H. Merritt, J. H. Hammond, W. Lu, J. D. Rabinowitz and G. A. O'Toole (2010). "Cyclic-di-GMP-Mediated Repression of Swarming Motility by *Pseudomonas aeruginosa*: the pilY1 Gene and Its Impact on Surface-Associated Behaviors." Journal of Bacteriology **192(12)**: 2950-2964.

Kuchma, S. L., K. M. Brothers, J. H. Merritt, N. T. Liberati, F. M. Ausubel and G. A. O'Toole (2007). "BifA, a Cyclic-Di-GMP Phosphodiesterase, Inversely Regulates Biofilm Formation and Swarming Motility by *Pseudomonas aeruginosa* PA14." Journal of Bacteriology **189(22)**: 8165-8178.

Kumar, C. G. and S. K. Mamidyala (2011). "Extracellular synthesis of silver nanoparticles using culture supernatant of *Pseudomonas aeruginosa*." Colloids and Surfaces B: Biointerfaces **84(2)**: 462-466.

Lai, C.-Y., L. Zhong, Y. Zhang, J.-X. Chen, L.-L. Wen, L.-D. Shi, Y.-P. Sun, F. Ma, B. E. Rittmann, C. Zhou, Y. Tang, P. Zheng and H.-P. Zhao (2016). "Bioreduction of Chromate in a Methane-Based Membrane Biofilm Reactor." Environmental Science & Technology **50(11)**: 5832-5839.

Lai, K. C. K. and I. M. C. Lo (2008). "Removal of Chromium (VI) by Acid-Washed Zero-Valent Iron under Various Groundwater Geochemistry Conditions." Environmental Science & Technology **42(4)**: 1238-1244.

Landini, P., D. Antoniani, J. G. Burgess and R. Nijland (2010). "Molecular mechanisms of compounds affecting bacterial biofilm formation and dispersal." Applied Microbiology and Biotechnology **86(3)**: 813-823.

Lange, A., G. C. Paull, T. S. Coe, Y. Katsu, H. Urushitani, T. Iguchi and C. R. Tyler (2009). "Sexual Reprogramming and Estrogenic Sensitization in Wild Fish Exposed to Ethinylestradiol." Environmental Science & Technology **43(4)**: 1219-1225.

Law, N., S. Ansari, F. R. Livens, J. C. Renshaw and J. R. Lloyd (2008). "Formation of Nanoscale Elemental Silver Particles via Enzymatic Reduction by *Geobacter sulfurreducens*." Applied and Environmental Microbiology **74(22)**: 7090-7093.

Lengke, M. F., M. E. Fleet and G. Southam (2007). "Biosynthesis of Silver Nanoparticles by Filamentous Cyanobacteria from a Silver(I) Nitrate Complex." Langmuir **23(5)**: 2694-2699.

Leonard, A. and R. Lauwerys (1980). "Carcinogenicity and mutagenicity of chromium." Mutation Research/Reviews in Genetic Toxicology **76(3)**: 227-239.

Li, X. Z., J. S. Webb, S. Kjelleberg and B. Rosche (2006). "Enhanced benzaldehyde tolerance in *Zymomonas mobilis* biofilms and the potential of biofilm applications in fine-chemical production." Applied and environmental microbiology **72(2)**: 1639-1644.

Li, Z., S.-W. Chung, J.-M. Nam, D. S. Ginger and C. A. Mirkin (2003). "Living Templates for the Hierarchical Assembly of Gold Nanoparticles." Angewandte Chemie **115(20)**: 2408-2411.

Lieleg, O., M. Caldara, R. Baumgartel and K. Ribbeck (2011). "Mechanical robustness of *Pseudomonas aeruginosa* biofilms." Soft Matter **7(7)**: 3307-3314.

Liu, C., Y. A. Gorby, J. M. Zachara, J. K. Fredrickson and C. F. Brown (2002). "Reduction kinetics of Fe(III), Co(III), U(VI), Cr(VI), and Tc(VII) in cultures of dissimilatory metal-reducing bacteria." Biotechnology and Bioengineering **80(6)**: 637-649.

Liu, H. and H. H. Fang (2002). "Extraction of extracellular polymeric substances (EPS) of sludges." Journal of Biotechnology **95(3)**: 249-256.

Liu, H., R. Ramnarayanan and B. E. Logan (2004). "Production of electricity during wastewater treatment using a single chamber microbial fuel cell." Environmental science & technology **38(7)**: 2281-2285.

Liu, Y. and J.-H. Tay (2002). "The essential role of hydrodynamic shear force in the formation of biofilm and granular sludge." Water Research **36(7)**: 1653-1665.

Lloyd, J. R., J. M. Byrne and V. S. Coker (2011). "Biotechnological synthesis of functional nanomaterials." Current Opinion in Biotechnology **22(4)**: 509-515.

Lloyd, J. R., P. Yong and L. E. Macaskie (1998). "Enzymatic Recovery of Elemental Palladium by Using Sulfate-Reducing Bacteria." Applied and Environmental Microbiology **64(11)**: 4607-4609.

Lovley, D. R. (1991). "Dissimilatory Fe(III) and Mn(IV) reduction." Microbiological Reviews **55(2)**: 259-287.

Lovley, D. R. (1993). "Dissimilatory metal reduction." Annual Reviews in Microbiology **47(1)**: 263-290.

Lovley, D. R. (2012). "Electromicrobiology." Annual review of microbiology **66**: 391-409.

Lovley, D. R. and R. T. Anderson (2000). "Influence of dissimilatory metal reduction on fate of organic and metal contaminants in the subsurface." Hydrogeology Journal **8(1)**: 77-88.

Lovley, D. R., J. D. Coates, E. L. Blunt-Harris, E. J. Phillips and J. C. Woodward (1996). "Humic substances as electron acceptors for microbial respiration." Nature **382(6590)**: 445-448.

Lovley, D. R., E. J. P. Phillips, Y. A. Gorby and E. R. Landa (1991). "Microbial reduction of uranium." Nature **350(6317)**: 413-416.

Magudapathy, P., P. Gangopadhyay, B. Panigrahi, K. Nair and S. Dhara (2001). "Electrical transport studies of Ag nanoclusters embedded in glass matrix." Physica B: Condensed Matter **299(1)**: 142-146.

Malvankar, N. S. and D. R. Lovley (2014). "Microbial nanowires for bioenergy applications." Current Opinion in Biotechnology **27**: 88-95.

Mandal, D., M. E. Bolander, D. Mukhopadhyay, G. Sarkar and P. Mukherjee (2006). "The use of microorganisms for the formation of metal nanoparticles and their application." Applied Microbiology and Biotechnology **69(5)**: 485-492.

Marsh, H. and F. R. Reinoso (2006). Activated carbon, Elsevier.

Marshall, M. J., A. S. Beliaev, A. C. Dohnalkova, D. W. Kennedy, L. Shi, Z. Wang, M. I. Boyanov, B. Lai, K. M. Kemner, J. S. McLean, S. B. Reed, D. E. Culley, V. L. Bailey, C. J. Simonson, D. A. Saffarini, M. F. Romine, J. M. Zachara and J. K. Fredrickson (2006). "c-Type cytochrome-dependent formation of U(IV) nanoparticles by *Shewanella oneidensis*." PLoS Biology **4(9)**: e268.

Marshall, M. J., A. E. Plymale, D. W. Kennedy, L. Shi, Z. Wang, S. B. Reed, A. C. Dohnalkova, C. J. Simonson, C. Liu, D. A. Saffarini, M. F. Romine, J. M. Zachara, A. S. Beliaev and J. K. Fredrickson (2008). "Hydrogenase- and outer membrane c-type cytochrome-facilitated reduction of technetium(VII) by *Shewanella oneidensis* MR-1." Environmental Microbiology **10(1)**: 125-136.

Marsili, E., D. B. Baron, I. D. Shikhare, D. Coursolle, J. A. Gralnick and D. R. Bond (2008). "Shewanella secretes flavins that mediate extracellular electron transfer." Proceedings of the National Academy of Sciences **105(10)**: 3968-3973.

Matveeva, V. G., P. M. Valetsky, M. G. Sulman, L. M. Bronstein, A. I. Sidorov, V. Y. Doluda, A. V. Gavrilenko, L. Z. Nikoshvili, A. V. Bykov, M. V. Grigorjev and E. M. Sulman (2011). "Nanosized Pt-, Ru-, and Pd-containing catalysts for organic synthesis and solution of environmental issues." Catalysis in Industry **3(3)**: 260-270.

McDougald, D., S. A. Rice, N. Barraud, P. D. Steinberg and S. Kjelleberg (2012). "Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal." Nat Rev Micro **10(1)**: 39-50.

McMurry, J. (1996). "Organic Chemistry, Brooks." Cole, New York: 657.

McSwain, B. S., R. L. Irvine, M. Hausner and P. A. Wilderer (2005). "Composition and Distribution of Extracellular Polymeric Substances in Aerobic Flocs and Granular Sludge." Applied and Environmental Microbiology **71(2)**: 1051-1057.

Melber, C., D. Keller and I. Mangelsdorf (2002). Environmental Health Criteria 226: Palladium. Geneva, World Health Organization.

Merritt, J. H., D. E. Kadouri and G. A. O'Toole (2005). Growing and Analyzing Static Biofilms. Current Protocols in Microbiology, John Wiley & Sons, Inc.

Meshulam-Simon, G., S. Behrens, A. D. Choo and A. M. Spormann (2007). "Hydrogen Metabolism in *Shewanella oneidensis* MR-1." Applied and Environmental Microbiology **73(4)**: 1153-1165.

Mikheenko, I. P., M. Rousset, S. Dementin and L. E. Macaskie (2008).

"Bioaccumulation of Palladium by *Desulfovibrio fructosivorans* Wild-Type and Hydrogenase-Deficient Strains." Applied and Environmental Microbiology **74(19)**: 6144-6146.

Milliken, C. and H. May (2007). "Sustained generation of electricity by the spore-forming, Gram-positive, *Desulfitobacterium hafniense* strain DCB2." Applied microbiology and biotechnology **73(5)**: 1180-1189.

Mohammed Fayaz, A., M. Girilal, M. Rahman, R. Venkatesan and P. T. Kalaichelvan (2011). "Biosynthesis of silver and gold nanoparticles using thermophilic bacterium *Geobacillus stearothermophilus*." Process Biochemistry **46(10)**: 1958-1962.

Morones, J. R., J. L. Elechiguerra, A. Camacho, K. Holt, J. B. Kouri, J. T. Ramírez and M. J. Yacaman (2005). "The bactericidal effect of silver nanoparticles." Nanotechnology **16(10)**: 2346.

Mortazavi, A., B. A. Williams, K. McCue, L. Schaeffer and B. Wold (2008). "Mapping and quantifying mammalian transcriptomes by RNA-Seq." Nature methods **5(7)**: 621-628.

Mulvaney, P. (1996). "Surface plasmon spectroscopy of nanosized metal particles." Langmuir **12(3)**: 788-800.

Myers, C. R. and K. H. Nealson (1988). "Bacterial Manganese Reduction and Growth with Manganese Oxide as the Sole Electron-Acceptor." Science **240(4857)**: 1319-1321.

Myers, C. R. and K. H. Nealson (1988). "Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor." Science **240**.

Nanda, A. and M. Saravanan (2009). "Biosynthesis of silver nanoparticles from *Staphylococcus aureus* and its antimicrobial activity against MRSA and MRSE." Nanomedicine: Nanotechnology, Biology and Medicine **5(4)**: 452-456.

Narayanan, K. B. and N. Sakthivel (2010). "Biological synthesis of metal nanoparticles by microbes." Advances in Colloid and Interface Science **156(1–2)**: 1-13.

Nealson, K. H. (1997). "Sediment bacteria: who's there, what are they doing, and what's new?" Annual Review of Earth and Planetary Sciences **25(1)**: 403-434.

Nealson, K. H. and S. E. Finkel (2011). "Electron flow and biofilms." MRS Bulletin **36(05)**: 380-384.

Nel, A., T. Xia, L. Mädler and N. Li (2006). "Toxic Potential of Materials at the Nanolevel." Science **311(5761)**: 622-627.

Ng, C. K., C. D. Bope, A. Nalaparaju, Y. Cheng, L. Lu, R. Wang and B. Cao (2017). "Concentrating synthetic estrogen 17 α -ethinylestradiol using microporous polyethersulfone hollow fiber membranes: Experimental exploration and molecular simulation." Chemical Engineering Journal **314**: 80-87.

Ng, C. K., T. K. Cai Tan, H. Song and B. Cao (2013). "Reductive formation of palladium nanoparticles by *Shewanella oneidensis*: role of outer membrane cytochromes and hydrogenases." RSC Advances **3(44)**: 22498-22503.

Ng, C. K. and B. Cao (2015). "What Exactly Are You Filtering Out?" Environmental Science & Technology **49(9)**: 5259-5260.

Ng, C. K., K. Sivakumar, X. Liu, M. Madhaiyan, L. Ji, L. Yang, C. Tang, H. Song, S. Kjelleberg and B. Cao (2013). "Influence of outer membrane c-type cytochromes on particle size and activity of extracellular nanoparticles produced by *Shewanella oneidensis*." Biotechnology and Bioengineering **110(7)**: 1831-1837.

Nguyen, H. D., B. Cao, B. Mishra, M. I. Boyanov, K. M. Kemner, J. K.

Fredrickson and H. Beyenal (2012). "Microscale geochemical gradients in Hanford 300 Area sediment biofilms and influence of uranium." Water Research **46(1)**: 227-234.

Niemeyer, C. M. (2001). "Nanoparticles, Proteins, and Nucleic Acids: Biotechnology Meets Materials Science." Angewandte Chemie International Edition **40(22)**: 4128-4158.

O'Toole, G. A. and R. Kolter (1998). "Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development." Molecular Microbiology **30(2)**: 295-304.

O'Brien, T. J., S. Ceryak and S. R. Patierno (2003). "Complexities of chromium carcinogenesis: role of cellular response, repair and recovery mechanisms." Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis **533(1-2)**: 3-36.

O'Loughlin, C. T., L. C. Miller, A. Siryaporn, K. Drescher, M. F. Semmelhack and B. L. Bassler (2013). "A quorum-sensing inhibitor blocks *Pseudomonas aeruginosa*

virulence and biofilm formation." Proceedings of the National Academy of Sciences **110(44)**: 17981-17986.

Ohko, Y., K.-i. Iuchi, C. Niwa, T. Tatsuma, T. Nakashima, T. Iguchi, Y. Kubota and A. Fujishima (2002). "17 β -Estradiol Degradation by TiO₂ Photocatalysis as a Means of Reducing Estrogenic Activity." Environmental Science & Technology **36(19)**: 4175-4181.

Okamoto, A., K. Hashimoto and R. Nakamura (2012). "Long-range electron conduction of *Shewanella* biofilms mediated by outer membrane C-type cytochromes." Bioelectrochemistry **85**: 61-65.

Omole, M. A., I. O. K'Owino and O. A. Sadik (2007). "Palladium nanoparticles for catalytic reduction of Cr(VI) using formic acid." Applied Catalysis B: Environmental **76(1–2)**: 158-167.

Owen, R. and S. Jobling (2012). "Environmental science: The hidden costs of flexible fertility." Nature **485(7399)**: 441-441.

Pal, S., Y. Tak and J. Song (2007). "Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the gram-negative bacterium *Escherichia coli*." Applied and Environmental Microbiology **73(6)**: 1712-1720.

Paraknowitsch, J. P. and A. Thomas (2013). "Doping carbons beyond nitrogen: an overview of advanced heteroatom doped carbons with boron, sulphur and phosphorus for energy applications." Energy & Environmental Science **6(10)**: 2839-2855.

Park, J., D.-H. Lim, H.-J. Lim, T. Kwon, J.-s. Choi, S. Jeong, I.-H. Choi and J. Cheon (2011). "Size dependent macrophage responses and toxicological effects of Ag nanoparticles." Chemical Communications **47(15)**: 4382-4384.

Park, J., D.-H. Lim, H.-J. Lim, T. Kwon, J. Choi, S. Jeong, I.-H. Choi and J. Cheon (2011). "Size dependent macrophage responses and toxicological effects of Ag nanoparticles." Chemical Communications **47**: 4382-4384.

Patey, G. M. and J. P. Valleau (1973). " The free energy of spheres with dipoles: Monte Carlo with multiscale sampling." J. Chem. Phys. Lett **21**.

Paul, K., V. Nieto, W. C. Carlquist, D. F. Blair and R. M. Harshey (2010). "The c-di-GMP Binding Protein YcgR Controls Flagellar Motor Direction and Speed to Affect Chemotaxis by a Backstop Brake Mechanism." Molecular cell **38(1)**: 128-139.

Perdrew, J. P. and Y. Wang (1992). "Accurate and simple analytic representation of the electron-gas correlation energy." J. Phys. Chem. B **45**: 13244 - 13249.

Perez-Alonso, F. J., D. N. McCarthy, A. Nierhoff, P. Hernandez-Fernandez, C. Strebel, I. E. Stephens, J. H. Nielsen and I. Chorkendorff (2012). "The effect of size on the oxygen electroreduction activity of mass-selected platinum nanoparticles." Angewandte Chemie **51(19)**: 4641-4643.

Perez-Alonso, F. J., D. N. McCarthy, A. Nierhoff, P. Hernandez-Fernandez, C. Strebel, I. E. L. Stephens, J. H. Nielsen and I. Chorkendorff (2012). "The Effect of Size on the Oxygen Electroreduction Activity of Mass-Selected Platinum Nanoparticles." Angewandte Chemie International Edition **51(19)**: 4641-4643.

- Pirbadian, S., S. E. Barchinger, K. M. Leung, H. S. Byun, Y. Jangir, R. A. Bouhenni, S. B. Reed, M. F. Romine, D. A. Saffarini, L. Shi, Y. A. Gorby, J. H. Golbeck and M. Y. El-Naggar** (2014). "Shewanella oneidensis MR-1 nanowires are outer membrane and periplasmic extensions of the extracellular electron transport components." Proceedings of the National Academy of Sciences.
- Ponder, S. M., J. G. Darab and T. E. Mallouk** (2000). "Remediation of Cr(VI) and Pb(II) Aqueous Solutions Using Supported, Nanoscale Zero-valent Iron." Environmental Science & Technology **34(12)**: 2564-2569.
- Powell, L. C., A. Sowedan, S. Khan, C. J. Wright, K. Hawkins, E. Onsøyen, R. Myrvold, K. E. Hill and D. W. Thomas** (2013). "The effect of alginate oligosaccharides on the mechanical properties of Gram-negative biofilms." Biofouling **29(4)**: 413-421.
- Pratt, L. A. and R. Kolter** (1998). "Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili." Molecular microbiology **30(2)**: 285-293.
- Prigent - Combaret, C., G. Prensier, T. T. Le Thi, O. Vidal, P. Lejeune and C. Dorel** (2000). "Developmental pathway for biofilm formation in curli - producing *Escherichia coli* strains: role of flagella, curli and colanic acid." Environmental microbiology **2(4)**: 450-464.
- Prouty, A. M., W. H. Schwesinger and J. S. Gunn** (2002). "Biofilm Formation and Interaction with the Surfaces of Gallstones by *Salmonella* spp." Infection and Immunity **70(5)**: 2640-2649.

Quintelas, C., B. Fernandes, J. Castro, H. Figueiredo and T. Tavares (2008).

"Biosorption of Cr(VI) by a *Bacillus coagulans* biofilm supported on granular activated carbon (GAC)." Chemical Engineering Journal **136(2–3)**: 195-203.

Quintelas, C., E. Sousa, F. Silva, S. Neto and T. Tavares (2006). "Competitive biosorption of ortho-cresol, phenol, chlorophenol and chromium(VI) from aqueous solution by a bacterial biofilm supported on granular activated carbon." Process Biochemistry **41(9)**: 2087-2091.

Rabaey, K., N. Boon, M. Höfte and W. Verstraete (2005). "Microbial phenazine production enhances electron transfer in biofuel cells." Environmental science & technology **39(9)**: 3401-3408.

Rakshe, S., M. Leff and A. M. Spormann (2011). "Indirect Modulation of the Intracellular c-Di-GMP Level in *Shewanella oneidensis* MR-1 by MxdA." Applied and Environmental Microbiology **77(6)**: 2196-2198.

Raman, D. R., E. L. Williams, A. C. Layton, R. T. Burns, J. P. Easter, A. S.

Daugherty, M. D. Mullen and G. S. Sayler (2004). "Estrogen Content of Dairy and Swine Wastes." Environmental Science & Technology **38(13)**: 3567-3573.

Raveendran, P., J. Fu and S. L. Wallen (2003). "Completely “Green” Synthesis and Stabilization of Metal Nanoparticles." Journal of the American Chemical Society **125(46)**: 13940-13941.

Reardon, C. L., A. C. Dohnalkova, P. Nachimuthu, D. W. Kennedy, D. A.

Saffarini, B. W. Arey, L. Shi, Z. Wang, D. Moore, J. S. McLean, D. Moyles, M. J.

Marshall, J. M. Zachara, J. K. Fredrickson and A. S. Beliaev (2010). "Role of

outer-membrane cytochromes MtrC and OmcA in the biomineralization of ferrihydrite by *Shewanella oneidensis* MR-1." Geobiology **8(1)**: 56-68.

Redwood, M. D., K. Deplanche, V. S. Baxter-Plant and L. E. Macaskie (2008).

"Biomass-supported palladium catalysts on *Desulfovibrio desulfuricans* and *Rhodobacter sphaeroides*." Biotechnology and Bioengineering **99(5)**: 1045-1054.

Reguera, G., K. P. Nevin, J. S. Nicoll, S. F. Covalla, T. L. Woodard and D. R.

Lovley (2006). "Biofilm and Nanowire Production Leads to Increased Current in *Geobacter sulfurreducens* Fuel Cells." Applied and Environmental Microbiology **72(11)**: 7345-7348.

Richardson, D. J., J. N. Butt, J. K. Fredrickson, J. M. Zachara, L. Shi, M. J.

Edwards, G. White, N. Baiden, A. J. Gates, S. J. Marritt and T. A. Clarke (2012).

"The 'porin-cytochrome' model for microbe-to-mineral electron transfer." Molecular Microbiology.

Richardson, D. J., M. J. Edwards, G. F. White, N. Baiden, R. S. Hartshorne, J.

Fredrickson, L. Shi, J. Zachara, A. J. Gates, J. N. Butt and T. A. Clarke (2012).

"Exploring the biochemistry at the extracellular redox frontier of bacterial mineral Fe(III) respiration." Biochemical Society Transactions **40(3)**: 493-500.

Riemer, J., H. H. Hoepken, H. Czerwinska, S. R. Robinson and R. Dringen

(2004). "Colorimetric ferrozine-based assay for the quantitation of iron in cultured cells." Analytical Biochemistry **331(2)**: 370-375.

Rodriguez, G. G., D. Phipps, K. Ishiguro and H. F. Ridgway (1992). "Use of a

fluorescent redox probe for direct visualization of actively respiring bacteria." Applied and Environmental Microbiology **58(6)**: 1801-1808.

Romero, D., C. Aguilar, R. Losick and R. Kolter (2010). "Amyloid fibers provide structural integrity to *Bacillus subtilis* biofilms." Proceedings of the National Academy of Sciences **107(5)**: 2230-2234.

Rosche, B., X. Z. Li, B. Hauer, A. Schmid and K. Buehler (2009). "Microbial biofilms: a concept for industrial catalysis?" Trends in biotechnology **27(11)**: 636-643.

Rosenfeldt, E. J. and K. G. Linden (2004). "Degradation of Endocrine Disrupting Chemicals Bisphenol A, Ethinyl Estradiol, and Estradiol during UV Photolysis and Advanced Oxidation Processes." Environmental Science & Technology **38(20)**: 5476-5483.

Rotaru, A. E., W. Jiang, K. Finster, T. Skrydstrup and R. L. Meyer (2012). "Non-enzymatic palladium recovery on microbial and synthetic surfaces." Biotechnology and Bioengineering.

Ryder, C., M. Byrd and D. J. Wozniak (2007). "Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development." Current opinion in microbiology **10(6)**: 644-648.

Ryu, J.-H. and L. R. Beuchat (2005). "Biofilm formation by *Escherichia coli* O157: H7 on stainless steel: effect of exopolysaccharide and curli production on its resistance to chlorine." Applied and Environmental Microbiology **71(1)**: 247-254.

Salnikow, K. and A. Zhitkovich (2008). "Genetic and Epigenetic Mechanisms in Metal Carcinogenesis and Cocarcinogenesis: Nickel, Arsenic, and Chromium." Chemical Research in Toxicology **21(1)**: 28-44.

Salta, M., J. A. Wharton, P. Stoodley, S. P. Dennington, L. R. Goodes, S.

Werwinski, U. Mart, R. J. K. Wood and K. R. Stokes (2010). "Designing biomimetic antifouling surfaces." Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences **368(1929)**: 4729-4754.

Sander, P., P. Szilard, S. Roland, L. Per, B. Par, A. Rossen, R. S. Michael, C. S.

Jeremy, P. M. Kasson, S. David van der, H. Berk and L. Erik (2013). "GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit." Bioinformatics **29**: 845-854.

Saville, R., S. Rakshe, J. Haagenen, S. Shukla and A. Spormann (2011).

"Energy-dependent Stability of *Shewanella oneidensis* MR-1 Biofilms." Journal of Bacteriology **193(13)**: 3253-3264.

Schäfer, A., L. Nghiem and T. Waite (2003). "Removal of the natural hormone estrone from aqueous solutions using nanofiltration and reverse osmosis."

Environmental Science & Technology **37(1)**: 182-188.

Schäfer, A. I., L. D. Nghiem and T. D. Waite (2003). "Removal of the Natural

Hormone Estrone from Aqueous Solutions Using Nanofiltration and Reverse Osmosis." Environmental Science & Technology **37(1)**: 182-188.

Scirè, S. and L. F. Liotta (2012). "Supported gold catalysts for the total oxidation of volatile organic compounds." Applied Catalysis B: Environmental **125**: 222-246.

Setiawan, L., R. Wang, K. Li and A. G. Fane (2012). "Fabrication and

characterization of forward osmosis hollow fiber membranes with antifouling NF-like selective layer." Journal of Membrane Science **394–395**: 80-88.

Shi, J., S. Fujisawa, S. Nakai and M. Hosomi (2004). "Biodegradation of natural and synthetic estrogens by nitrifying activated sludge and ammonia-oxidizing bacterium *Nitrosomonas europaea*." Water Research **38(9)**: 2323-2330.

Shi, L., S. M. Belchik, A. E. Plymale, S. Heald, A. C. Dohnalkova, K. Sybirna, H. Bottin, T. C. Squier, J. M. Zachara and J. K. Fredrickson (2011). "Purification and characterization of the [NiFe]-Hydrogenase of *Shewanella oneidensis* MR-1." Applied and Environmental Microbiology **77(16)**: 5584-5590.

Shi, L., B. Chen, Z. Wang, D. A. Elias, M. U. Mayer, Y. A. Gorby, S. Ni, B. H. Lower, D. W. Kennedy, D. S. Wunschel, H. M. Mottaz, M. J. Marshall, E. A. Hill, A. S. Beliaev, J. M. Zachara, J. K. Fredrickson and T. C. Squier (2006). "Isolation of a High-Affinity Functional Protein Complex between OmcA and MtrC: Two Outer Membrane Decaheme c-Type Cytochromes of *Shewanella oneidensis* MR-1." Journal of Bacteriology **188(13)**: 4705-4714.

Shi, L., H. Dong, G. Reguera, H. Beyenal, A. Lu, J. Liu, H.-Q. Yu and J. K. Fredrickson (2016). "Extracellular electron transfer mechanisms between microorganisms and minerals." Nat Rev Micro **14(10)**: 651-662.

Shi, L., D. J. Richardson, Z. Wang, S. Kerisit, K. M. Rosso, J. M. Zachara and J. K. Fredrickson (2009). "The roles of outer membrane cytochromes of *Shewanella* and *Geobacter* in extracellular electron transfer." Environmental Microbiology Reports **1(4)**: 220-227.

Shi, L., K. M. Rosso, T. A. Clarke, D. J. Richardson, J. M. Zachara and J. K. Fredrickson (2012). "Molecular Underpinnings of Fe(III) Oxide Reduction by *Shewanella oneidensis* MR-1." Frontiers in Microbiology **3**: 50.

Shi, L., T. C. Squier, J. M. Zachara and J. K. Fredrickson (2007). "Respiration of metal (hydr)oxides by *Shewanella* and *Geobacter*: a key role for multihaem c-type cytochromes." Molecular Microbiology **65(1)**: 12-20.

Simm, R., M. Morr, A. Kader, M. Nimtz and U. Römling (2004). "GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility." Molecular Microbiology **53(4)**: 1123-1134.

Simões, M., L. C. Simões and M. J. Vieira (2010). "A review of current and emergent biofilm control strategies." LWT - Food Science and Technology **43(4)**: 573-583.

Singh, P. K., A. L. Schaefer, M. R. Parsek, T. O. Moninger, M. J. Welsh and E. P. Greenberg (2000). "Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms." Nature **407(6805)**: 762-764.

Singh, R., D. Paul and R. K. Jain (2006). "Biofilms: implications in bioremediation." Trends in microbiology **14(9)**: 389-397.

Sintubin, L., W. De Windt, J. Dick, J. Mast, D. van der Ha, W. Verstraete and N. Boon (2009). "Lactic acid bacteria as reducing and capping agent for the fast and efficient production of silver nanoparticles." Applied Microbiology and Biotechnology **84(4)**: 741-749.

Sintubin, L., W. Verstraete and N. Boon (2012). "Biologically produced nanosilver: Current state and future perspectives." Biotechnology and Bioengineering.

Sivakumar, K., M. Mukherjee, C. Hsin - I, Z. Yingdan, L. Ji and B. Cao (2014). "Surface display of roGFP for monitoring redox status of extracellular

microenvironments in *Shewanella oneidensis* biofilms." Biotechnology and bioengineering.

Skolimowski, M., M. W. Nielsen, J. Emneus, S. Molin, R. Taboryski, C.

Sternberg, M. Dufva and O. Geschke (2010). "Microfluidic dissolved oxygen gradient generator biochip as a useful tool in bacterial biofilm studies." Lab on a Chip **10(16)**: 2162-2169.

Snyder, S. A., D. L. Villeneuve, E. M. Snyder and J. P. Giesy (2001).

"Identification and Quantification of Estrogen Receptor Agonists in Wastewater Effluents." Environmental Science & Technology **35(18)**: 3620-3625.

Sobjerg, L. S., D. Gauthier, A. T. Lindhardt, M. Bunge, K. Finster, R. L. Meyer and T. Skrydstrup (2009). "Bio-supported palladium nanoparticles as a catalyst for Suzuki-Miyaura and Mizoroki-Heck reactions." Green Chemistry **11(12)**: 2041-2046.

Søbjerg, L. S., A. T. Lindhardt, T. Skrydstrup, K. Finster and R. L. Meyer (2011).

"Size control and catalytic activity of bio-supported palladium nanoparticles." Colloids and Surfaces B: Biointerfaces **85(2)**: 373-378.

Spurbeck, R. R., R. J. Tarrien and H. L. T. Mobley (2012). "Enzymatically Active and Inactive Phosphodiesterases and Diguanylate Cyclases Are Involved in Regulation of Motility or Sessility in *Escherichia coli* CFT073." mBio **3(5)**.

Srivastava, S. K. and M. Constanti (2012). "Room temperature biogenic synthesis of multiple nanoparticles (Ag, Pd, Fe, Rh, Ni, Ru, Pt, Co, and Li) by *Pseudomonas aeruginosa* SM1." Journal of Nanoparticle Research **14(4)**: 831.

Sternberg, C. and T. Tolker-Nielsen (2006). "Growing and analyzing biofilms in flow cells." Current protocols in microbiology Chapter 1: Unit 1B 2.

Stewart, P. S. and M. J. Franklin (2008). "Physiological heterogeneity in biofilms." Nat Rev Micro **6(3)**: 199-210.

Stoodley, P., Z. Lewandowski, J. D. Boyle and H. M. Lappin-Scott (1998).

"Oscillation characteristics of biofilm streamers in turbulent flowing water as related to drag and pressure drop." *Biotechnology and Bioengineering* **57(5)**: 536-544.

Stubblefield, B. A., K. E. Howery, B. N. Islam, A. J. Santiago, W. E. Cardenas and E. S. Gilbert (2010). "Constructing multispecies biofilms with defined compositions by sequential deposition of bacteria." *Applied Microbiology and Biotechnology* **86(6)**: 1941-1946.

Sunkar, S. and C. V. Nachiyar (2012). "Biogenesis of antibacterial silver nanoparticles using the endophytic bacterium *Bacillus cereus* isolated from *Garcinia xanthochymus*." *Asian Pacific Journal of Tropical Biomedicine* **2(12)**: 953-959.

Suresh, A. K., D. A. Pelletier, W. Wang, M. L. Broich, J. W. Moon, B. Gu, D. P. Allison, D. C. Joy, T. J. Phelps and M. J. Doktycz (2011). "Biofabrication of discrete spherical gold nanoparticles using the metal-reducing bacterium *Shewanella oneidensis*." *Acta Biomaterialia* **7(5)**: 2148-2152.

Suresh, A. K., D. A. Pelletier, W. Wang, J.-W. Moon, B. Gu, N. P. Mortensen, D. P. Allison, D. C. Joy, T. J. Phelps and M. J. Doktycz (2010). "Silver Nanocrystallites: Biofabrication using *Shewanella oneidensis*, and an Evaluation of Their Comparative Toxicity on Gram-negative and Gram-positive Bacteria." *Environmental Science & Technology* **44(13)**: 5210-5215.

Suri, R. P. S., T. S. Singh and S. Abburi (2010). "Influence of Alkalinity and Salinity on the Sonochemical Degradation of Estrogen Hormones in Aqueous Solution." *Environmental Science & Technology* **44(4)**: 1373-1379.

Teixeira, M., I. Moura, A. V. Xavier, J. J. Moura, J. LeGall, D. V. DerVartanian, H. D. Peck and B. H. Huynh (1989). "Redox intermediates of Desulfovibrio gigas [NiFe] hydrogenase generated under hydrogen. Mössbauer and EPR characterization of the metal centers." *Journal of Biological Chemistry* **264(28)**: 16435-16450.

Templeton, A. S., T. P. Trainor, S. J. Traina, A. M. Spormann and G. E. Brown (2001). "Pb(II) distributions at biofilm–metal oxide interfaces." *Proceedings of the National Academy of Sciences* **98(21)**: 11897-11902.

Theunissen, S., L. De Smet, A. Dansercoer, B. Motte, T. Coenye, J. J. Van Beeumen, B. Devreese, S. N. Savvides and B. Vergauwen (2010). "The 285 kDa Bap/RTX hybrid cell surface protein (SO4317) of *Shewanella oneidensis* MR-1 is a key mediator of biofilm formation." *Research in microbiology* **161(2)**: 144-152.

Thormann, K. M., S. Duttler, R. M. Saville, M. Hyodo, S. Shukla, Y. Hayakawa and A. M. Spormann (2006). "Control of Formation and Cellular Detachment from *Shewanella oneidensis* MR-1 Biofilms by Cyclic di-GMP." *Journal of Bacteriology* **188(7)**: 2681-2691.

Thorpe, K. L., R. I. Cummings, T. H. Hutchinson, M. Scholze, G. Brighty, J. P. Sumpter and C. R. Tyler (2003). "Relative Potencies and Combination Effects of Steroidal Estrogens in Fish." *Environmental Science & Technology* **37(6)**: 1142-1149.

- Thorpe, K. L., G. Maack, R. Benstead and C. R. Tyler** (2009). "Estrogenic Wastewater Treatment Works Effluents Reduce Egg Production in Fish." Environmental Science & Technology **43(8)**: 2976-2982.
- Torrie, G. M. and J. P. Valleau** (1974). "Monte Carlo free energy estimates using Non-Boltzmann sampling application to the sub-critical Lennard-Jones fluid." J. Chem. Phys. Lett **28**.
- Torrie, G. M. and J. P. Valleau** (1977). "Nonphysical sampling distributions in Monte carlo free-energy estimation- Umbrella sampling." J. Comput. Phys. **23**: 187-199.
- Tsoligkas, A., M. Winn, J. Bowen, T. Overton, M. Simmons and R. Goss** (2011). "Engineering Biofilms for Biocatalysis." ChemBioChem **12**: 1391-1395.
- Valladares Linares, R., V. Yangali-Quintanilla, Z. Li and G. Amy** (2011). "Rejection of micropollutants by clean and fouled forward osmosis membrane." Water Research **45(20)**: 6737-6744.
- Vardanyan, Z. and A. Trchounian** (2010). "The Effects of Copper (II) Ions on Enterococcus hirae Cell Growth and the Proton-Translocating FoF1 ATPase Activity." Cell Biochemistry and Biophysics **57(1)**: 19-26.
- Von Canstein, H., J. Ogawa, S. Shimizu and J. R. Lloyd** (2008). "Secretion of flavins by Shewanella species and their role in extracellular electron transfer." Applied and environmental microbiology **74(3)**: 615-623.
- Wang, H., N. Law, G. Pearson, B. E. van Dongen, R. M. Jarvis, R. Goodacre and J. R. Lloyd** (2010). "Impact of Silver(I) on the Metabolism of *Shewanella oneidensis*." Journal of Bacteriology **192(4)**: 1143-1150.

Wang, L., N. Wang, L. Zhu, H. Yu and H. Tang (2008). "Photocatalytic reduction of Cr(VI) over different TiO₂ photocatalysts and the effects of dissolved organic species." Journal of Hazardous Materials **152(1)**: 93-99.

Wang, R., L. Shi, C. Y. Tang, S. Chou, C. Qiu and A. G. Fane (2010). "Characterization of novel forward osmosis hollow fiber membranes." Journal of Membrane Science **355(1-2)**: 158-167.

Wang, X., J. F. Preston and T. Romeo (2004). "The pgaABCD locus of *Escherichia coli* promotes the synthesis of a polysaccharide adhesin required for biofilm formation." Journal of bacteriology **186(9)**: 2724-2734.

Watnick, P. I., C. M. Lauriano, K. E. Klose, L. Croal and R. Kolter (2001). "The absence of a flagellum leads to altered colony morphology, biofilm development and virulence in *Vibrio cholerae* O139." Molecular microbiology **39(2)**: 223-235.

Wilkinson, D. A., S. J. Chacko, C. Vénien-Bryan, G. H. Wadhams and J. P. Armitage (2011). "Regulation of Flagellum Number by FliA and FlgM and Role in Biofilm Formation by *Rhodobacter sphaeroides*." Journal of Bacteriology **193(15)**: 4010-4014.

Wimpenny, J., W. Manz and U. Szewzyk (2000). "Heterogeneity in biofilms." FEMS Microbiology Reviews **24(5)**: 661-671.

Windt, W. D., P. Aelterman and W. Verstraete (2005). "Bioreductive deposition of palladium (0) nanoparticles on *Shewanella oneidensis* with catalytic activity towards reductive dechlorination of polychlorinated biphenyls." Environmental Microbiology **7(3)**: 314-325.

Winn, M., J. Foulkes, S. Perni, M. Simomons, T. Overton and R. Goss (2012).

"Biofilms and their engineered counterparts: A new generation of immobilised biocatalysts." Catalysis Science & Technology **2**: 1544-1547.

Wolfe, A. J. and K. L. Visick (2008). "Get the Message Out: Cyclic-Di-GMP Regulates Multiple Levels of Flagellum-Based Motility." Journal of Bacteriology **190(2)**: 463-475.

Woodley, J. M. (2006). "Microbial biocatalytic processes and their development." Advances in Applied Microbiology, Vol 60 **60**: 1-15.

Wu, G., R. Swaidan, D. Li and N. Li (2008). "Enhanced methanol electro-oxidation activity of PtRu catalysts supported on heteroatom-doped carbon." Electrochimica Acta **53(26)**: 7622-7629.

Wuertz, S., P. Bishop and P. Wilderer, Eds. (2003). Biofilms in Wastewater Treatment: An Interdisciplinary Approach. London, IWA Publishing.

Wuertz, S., P. Pfeleiderer, K. Kriebitzsch, R. Späth, T. Griebe, D. Coello-Oviedo, P. A. Wilderer and H.-C. Flemming (1998). "Extracellular redox activity in activated sludge." Water Science and Technology **37(4-5)**: 379-384.

Wuertz, S., R. Spaeth, A. Hinderberger, T. Griebe, H. Flemming and P. Wilderer (2001). "A new method for extraction of extracellular polymeric substances from biofilms and activated sludge suitable for direct quantification of sorbed metals." Water Science & Technology **43(6)**: 25-31.

- Xu, N., A. C. Johnson, M. D. Jürgens, N. R. Llewellyn, N. P. Hankins and R. C. Darton** (2009). "Estrogen Concentration Affects its Biodegradation Rate in Activated Sludge." Environmental Toxicology and Chemistry **28(11)**: 2263-2270.
- Yan, L., Y. S. Li and C. B. Xiang** (2005). "Preparation of poly(vinylidene fluoride)(pvdf) ultrafiltration membrane modified by nano-sized alumina (Al₂O₃) and its antifouling research." Polymer **46(18)**: 7701-7706.
- Yang, L., S. Jiang, Y. Zhao, L. Zhu, S. Chen, X. Wang, Q. Wu, J. Ma, Y. Ma and Z. Hu** (2011). "Boron-Doped Carbon Nanotubes as Metal-Free Electrocatalysts for the Oxygen Reduction Reaction." Angewandte Chemie **123(31)**: 7270-7273.
- Yates, M. D., R. D. Cusick, I. Ivanov and B. E. Logan** (2014). "Exoelectrogenic biofilm as a template for sustainable formation of a catalytic mesoporous structure." Biotechnology and Bioengineering **(11)**: 2349-2354.
- Yates, M. D., R. D. Cusick and B. E. Logan** (2013). "Extracellular Palladium Nanoparticle Production using *Geobacter sulfurreducens*." ACS Sustainable Chemistry & Engineering.
- Yates, M. D. and B. E. Logan** (2014). "Biotemplated Palladium Catalysts Can Be Stabilized on Different Support Materials." ChemElectroChem **1(11)**: 1867-1873.
- Yong, P., N. A. Rowson, J. P. G. Farr, I. R. Harris and L. E. Macaskie** (2002). "Bioreduction and biocrystallization of palladium by *Desulfovibrio desulfuricans* NCIMB 8307." Biotechnology and Bioengineering **80(4)**: 369-379.

- Yoon, Y., P. Westerhoff, S. A. Snyder and M. Esparza** (2003). "HPLC-fluorescence detection and adsorption of bisphenol A, 17 β -estradiol, and 17 α -ethynyl estradiol on powdered activated carbon." Water Research **37(14)**: 3530-3537.
- Zhang, H., Q. Li, Y. Lu, D. Sun, X. Lin, X. Deng, N. He and S. Zheng** (2005). "Biosorption and bioreduction of diamine silver complex by *Corynebacterium*." Journal of Chemical Technology & Biotechnology **80(3)**: 285-290.
- Zhang, Y., C. K. Ng, Y. Cohen and B. Cao** (2014). "Cell Growth and Protein Expression of *Shewanella oneidensis* in Biofilms and Hydrogel-entrapped Cultures." Molecular BioSystems.
- Zheng, H., J. Kim, M. Liew, John K. Yan, O. Herrera, Jin W. Bok, Neil L. Kelleher, Nancy P. Keller and Y. Wang** (2015). "Redox Metabolites Signal Polymicrobial Biofilm Development via the NapA Oxidative Stress Cascade in *Aspergillus*." Current Biology **25(1)**: 29-37.
- Zhou, Y., K. Neyerlin, T. S. Olson, S. Pylypenko, J. Bult, H. N. Dinh, T. Gennett, Z. Shao and R. O'Hayre** (2010). "Enhancement of Pt and Pt-alloy fuel cell catalyst activity and durability via nitrogen-modified carbon supports." Energy & Environmental Science **3(10)**: 1437-1446.
- Zhu, H., J. Yin, X. Wang, H. Wang and X. Yang** (2013). "Microorganism-Derived Heteroatom-Doped Carbon Materials for Oxygen Reduction and Supercapacitors." Advanced Functional Materials **23(10)**: 1305-1312.
- Zimmermann, K.** (1991). "ORAL: All Purpose Molecular Mechanics Simulator and Energy Minimizer." J. Comput. Chem **12**: 310-319.