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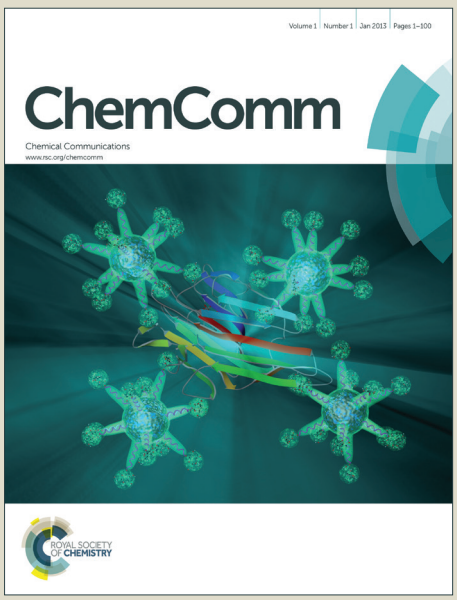

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Uncovering alternate charge transfer mechanisms in *Escherichia coli* chemically functionalized with conjugated oligoelectrolytes

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Integration of conjugated oligoelectrolytes in *Escherichia coli* has been proposed to act as 'molecular wires', inducing extracellular electron transfer. This is monitored through reduction of $HAuCl_4$ to form gold nanoparticles. In contrast to direct electron transfer, the reduction mechanism was traced to release of electroactive cytosolic components during oligoelectrolyte incorporation.

Microbial fuel cells (MFCs) exploit extracellular electron transfer (EET) mechanisms in microorganisms to concurrently produce energy and bioremediate waste contaminants.[1-5] In electrochemically active microbes, which can be employed in MFCs, EET occurs through various mechanisms – membrane associated cytochromes[6], secretion of soluble redox mediators[7, 8], and physical appendages such as conductive pili.[9, 10] Recent advances in this field have shown that microorganisms can be chemically functionalized by incorporating conjugated oligoelectrolytes into the microbial membrane.[11-15] These molecules consist of electronically delocalized π-conjugated aromatic backbones, while pendant ionic groups impart solubility in aqueous media. The amphiphilic nature allows insertion and alignment within lipid membranes. Intercalation of 4,4'-bis(4'-(*N*,*N*-bis(6"-(*N*,*N*,*N*-trimethylammonium)hexyl)amino)-styryl)stilbene tetraiodide (DSSN+) into the microbial membrane of *Escherichia coli*[15] and in yeast cells[11] resulted in MFCs with improved charge collection. Using a series of conjugated oligoelectrolytes to chemically modify *E. coli*, *Shewanella oneidensis*, enhanced electrical output, formation of reduced metabolites and wastewater bioremediation have been demonstrated.[11-15] The intercalated conjugated oligoelectrolyte has been proposed to act as a transmembrane electron transfer molecule.

Various EET mechanisms can be studied by using it to drive bio-reduction to form nanoparticles. The outer membrane c-type cytochromes of *S. oneidensis* have been shown to influence the size of extracellular silver and silver sulfide nanoparticles.[17] Nanoparticle location was used to determine the reduction mechanism with extracellular nanoparticle formation occurring when transmembrane electron transfer mechanisms are dominant.[18] Here, DSSN+ modified *E. coli* has been examined for bio-reduction by EET to form extracellular gold nanoparticles. Through control experiments, chemical transformation of $HAuCl_4$ to gold nanoparticles via leaked cytosolic components from the perturbation of microbial membrane by DSSN+ insertion is suggested. No excretion of electroactive cytosolic components was observed without DSSN+. This work uncovers an alternate mechanism to explain enhanced electrical performance in bioelectrochemical systems employing chemically modified *E. coli*.

DSSN+ was spontaneously incorporated into *E. coli* membrane after an incubation period of 5 hours and subsequently introduced to the reaction tubes containing $HAuCl_4$.[15] Within 15 minutes, purple precipitates were observed in the reaction tube containing *E. coli*, DSSN+ and $HAuCl_4$ (Fig. S1). The inset of Fig. 1A compares the reaction tube containing $HAuCl_4$ with unmodified *E. coli* (1) and DSSN+ incorporated *E. coli* (2). No colour change was observed in the control reaction tubes without DSSN+, indicating absence of any reaction. Optical absorption spectroscopy revealed the characteristic plasmonic absorption peak at ~550 nm (Fig. 1A). FESEM images indicated extracellular formation of discrete nanoparticles in the size regime of ~50 nm (Fig. 1B). Spot-profile energy dispersive X-ray (EDX) analysis (data not shown) reconfirmed them as gold nanoparticles. Control reaction tubes containing mixtures of DSSN+ and $HAuCl_4$ as well as $HAuCl_4$ alone in ultrapure water did not result in any reaction (Fig. S1). This shows that DSSN+ has to interact with *E. coli* to facilitate reduction of $AuCl_4^-$ ions to form gold nanoparticles.

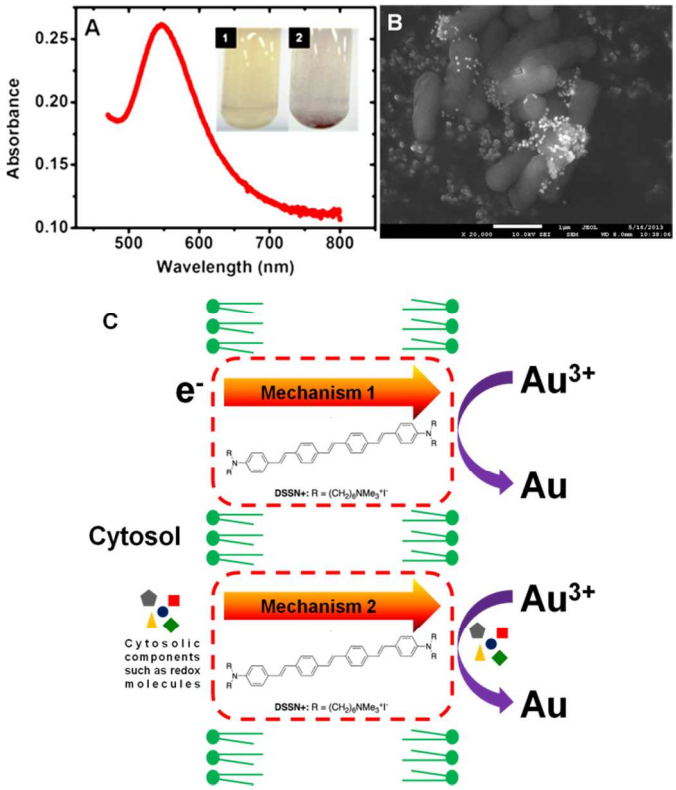

Fig. 1 (A) Optical absorption spectroscopy of nanoparticles formed in external aqueous media by bio-reduction of $HAuCl_4$ via interaction of DSSN+ with *E. coli*. The inset photographs depict reaction tubes with unmodified *E. coli* (1) and DSSN+ incorporated *E. coli* (2) after 4 hours. (B) Field emission scanning electron microscopy images of gold nanoparticles formed via interaction of DSSN+ with *E. coli* at 20 000× magnification after 4 hours. (C) Proposed EET pathways by chemically functionalized *E. coli* for chemical transformation of $HAuCl_4$ to gold nanoparticles. Chemical structure of DSSN+ is shown in the hatched box.

Two possible pathways exist for the extracellular formation of gold nanoparticles by DSSN+ incorporated *E. coli* (Fig. 1C). One pathway is through EET via incorporated DSSN+ (Mechanism 1, Fig. 1C). The second possible pathway is through the release of cytosolic components, such as redox molecules[19, 20], during DSSN+ incorporation (Mechanism 2, Fig. 1C). Membrane perturbation could occur during DSSN+ incorporation, leading to the release of these cytosolic components. To resolve the mechanism driving the reduction, reaction tubes with DSSN+ incorporated *E. coli* was centrifuged to isolate possible cytosolic components released into the supernatant. The centrifuged *E. coli*/DSSN+ pellet was thoroughly washed, re-suspended in sterile ultrapure water and homogenized. The addition of $HAuCl_4$ to these isolated *E. coli*/DSSN+ cells did not yield any gold nanoparticles (Fig. 2A), indicating absence of the reduction reaction. However, the supernatant (without DSSN+ incorporated *E. coli* cells) yielded dark purple precipitates when $HAuCl_4$ was added, (Fig. 2B), indicating that the leaked cytosolic components can function as reducing agents to form gold nanoparticle. Thus, the reduction was primarily driven by cytosolic components released into the supernatant, rather than DSSN+ incorporated *E. coli*. Previous studies show that metallic biotransformation can be driven by cellular redox molecules, such as quinones[21], which can reduce metals or metal oxides.

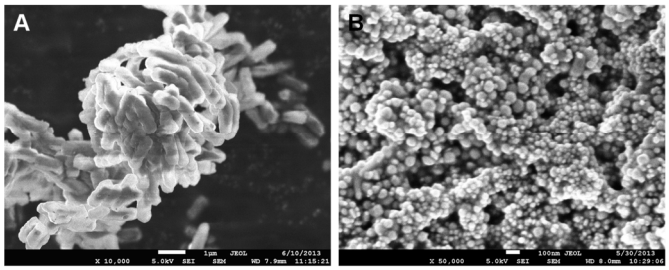

Fig. 2 (A) Field emission scanning electron microscopy image of re-suspended *E. coli*/DSSN+ pellet showing absence of nanoparticle formation. (B) Field emission scanning electron microscopy image of agglomerated nanoparticles collected from reaction tube containing supernatant and $HAuCl_4$ precursor.

In order to mimic release of cytosolic compounds during DSSN+ incorporation, ultra-sonication of *E. coli* culture was performed to intentionally rupture the cell membrane. No DSSN+ was introduced at this point. The ultra-sonicated mixture was centrifuged to isolate the released cytosolic components in the supernatant and $HAuCl_4$ subsequently introduced. Precipitates were again observed in the supernatant collected after sonication (Fig. S2A), whereas no change was observed in the control reaction tube (Fig. S2B) containing supernatant isolated from unsonicated *E. coli.* The obtained gold nanoparticles are 50 - 100 nm in size and appeared purple in colour. This indicates that the cytosolic components are only released during membrane perturbation processes (ultra-sonication or DSSN+ incorporation) which is similar to previous reports of enhanced *E. coli* activity through excretion of electroactive compounds after electrochemical activation[22] and time-evolved characterization at the genomic level.[23] These observations support our hypothesis that it is the released cytosolic components, such as redox molecules, that drive the chemical transformation of $AuCl_4^-$ ions. The presence of electroactive redox components in the solution was confirmed by cyclic voltammetry (CV) on supernatants from untreated *E. coli* which functioned as a control, separate ultra-sonication and DSSN+ incorporation processes (Fig. S3). CV traces indicate redox processes at ~-270 mV (vs Ag/AgCl).[24, 25] This suggests presence of electroactive components, such as quinones, which is abundant in *E. coli* cytosol.[19, 26] Comparable redox potentials for supernatants obtained from the separate processes of ultra-sonication and DSSN+ incorporation suggest that both systems release similar cytosolic components after membrane disruption. These redox potentials differ from those reported for DSSN+[11] (~+550 mV, ~+600 mV and ~+800 mV vs Ag/AgCl), indicating that DSSN+ did not contribute to the revealed redox potentials.

To check whether such released cytosolic components could generate signals in bioelectrochemical systems, MFCs *without microorganisms* were employed (Fig. 3A). DSSN+ incorporated MFCs typically show low electrical output in the range of few μA cm^{-2} (1-3 μA cm^{-2}) due to high internal resistance.[11, 12, 14, 15] Following procedures outlined above, supernatants were separately prepared from DSSN+ incorporation and ultra-sonication processes. MFCs employing washed and re-suspended DSSN+ incorporated *E. coli* generated negligible current density (black trace, Fig. 3B). However, the isolated supernatant from DSSN+ incubation generated a maximum current density of ~0.04 μA cm^{-2}, which stabilized to ~0.02 μA cm^{-2} (red trace, Fig. 3B). Similarly, the supernatant from ultra-sonication generated a maximum current density of ~0.14 μA cm^{-2} (subsequently stabilizing to ~0.10 μA cm^{-2}) (orange trace, Fig. 3B). The higher current density recorded for ultra-sonication as compared to DSSN+ incorporation is possibly due to the increased concentration of released cytosolic components.

These released cytosolic components contribute to the measured currents – possibly through their electrochemical oxidation at the electrode. Contrastingly, thoroughly washed DSSN+ incorporated *E. coli* did not produce any electrical signals. The minimum inhibitory and minimum biocidal concentrations of DSSN+ with *E. coli* are 64 μM and 128 μM respectively.[15] Although the working concentration for this study is 5 μM, a certain degree of membrane perturbation is expected. However due to low concentrations, the amount of released cytosolic components is low, directly affecting the current density recorded in the MFC platform. Therefore, it is possible that in previously reported cases of DSSN+ incorporation into *E. coli*[11-15], the released cytosolic components could have contributed a significant fraction of the electrical output in MFCs.

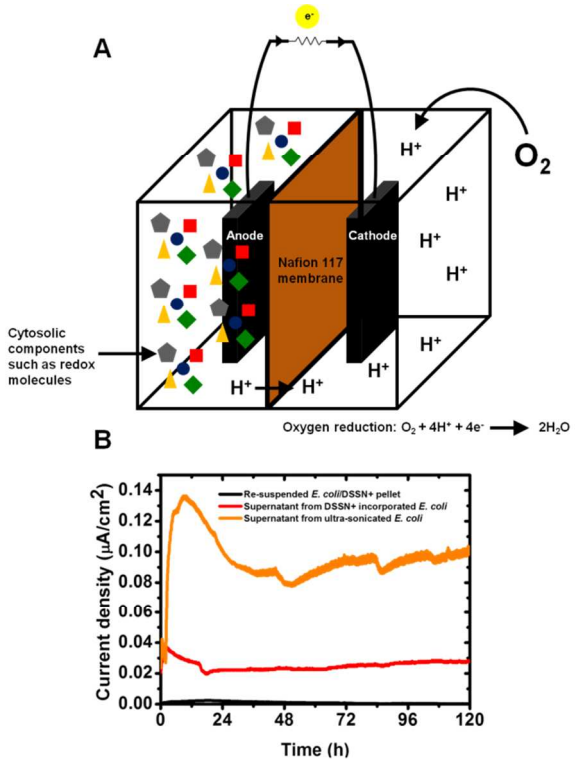

Fig. 3 (A) Schematic of MFC setup *without microorganisms*. (B) Current density versus time traces of MFCs with various forms of supernatant and re-suspended *E. coli*/DSSN+ cells. The black trace corresponds to re-suspended *E. coli*/DSSN+ pellet. The red and orange traces correspond to supernatants obtained after separate DSSN+ incorporation and ultra-sonication of *E. coli* respectively.

These findings strongly suggest that the bio-reduction of $AuCl_4^-$ ions was due to released cytosolic components via membrane perturbation, and not by transmembrane charge transfer. Although DSSN+ can spontaneously intercalate in *E. coli* cell membrane due to its amphiphilic nature, the mechanical rigidity and limited molecular flexibility of its backbone afforded by its specific core backbone moiety could perturb the membrane and damage the structural integrity.[27] Although the similarity of DSSN+ molecular length (~3.8-4 nm) to that of *E. coli* cell membrane thickness (~4 nm) [28] has been used to argue for EET, the length of the conducting core (~1.8-2.1 nm) is much shorter. The molecule further constitutes long insulating alkyl chains at the terminal ends of the molecule (Fig. S4), which do not contribute to charge transfer. The length mismatch may therefore impede transmembrane electron transfer. It should be noted that the destabilizing effects noted in the present study are at concentrations deemed suitable by a previous study on mammalian cells.[16] This highlights the need to specifically design molecules as well as determine appropriate intercalation conditions for the microbes employed in MFCs.

In conclusion, the release of cytosolic components from chemically modified *E. coli* primarily drove reductive transformation of $HAuCl_4$ to gold nanoparticles. Compromising structural integrity of the membrane by DSSN+ incorporation caused leakage of cytosolic components through the microbial membrane. Cyclic voltammetry data of DSSN+ incorporated and ultra-sonicated systems revealed release of similar cytosolic components. Further, these components contributed to electrical signals in a MFC configuration. Based on our findings, the enhanced performance observed in chemically modified *E. coli* could arise from microbial membrane perturbation.

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Notes and references

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1. D. R. Lovley, *Nat. Rev. Microbiol.*, 2006, **4**, 497-508.
2. K. Rabaey and W. Verstraete, *Trends biotechnol.*, 2005, **23**, 291-298.
3. L. Huang and B. Logan, *Appl. Microbiol. Biotech.*, 2008, **80**, 349-355.
4. Z. Du, H. Li and T. Gu, *Biotechnol. Adv.*, 2007, **25**, 464-482.
5. W.-W. Li and H.-Q. Yu, *Environ. Sci. Technol.*, 2013, **48**, 17-18.
6. D. R. Lovley, D. E. Holmes and K. P. Nevin, in *Adv. Microb. Physiol.*, Academic Press, 2004, vol. Volume 49, pp. 219-286.
7. D. H. Park and J. G. Zeikus, *Appl. Environ. Microb.*, 2000, **66**, 1292-1297.
8. K. Rabaey, N. Boon, M. Höfte and W. Verstraete, *Environ. Sci. Technol.*, 2005, **39**, 3401-3408.
9. G. Reguera, K. P. Nevin, J. S. Nicoll, S. F. Covalla, T. L. Woodard and D. R. Lovley, *Appl. Environ. Microbiol.*, **72**, 7345-7348.
10. Y. A. Gorby, 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, *Proc. Natl. Acad. Sci.*, 2006, **103**, 11358-11363.
11. L. E. Garner, J. Park, S. M. Dyar, A. Chworos, J. J. Sumner and G. C. Bazan, *J. Am. Chem. Soc.*, 2010, **132**, 10042-10052.
12. L. E. Garner, A. W. Thomas, J. J. Sumner, S. P. Harvey and G. C. Bazan, *Energ. Environ. Sci.*, 2012, **5**, 9449-9452.

13. H. Hou, X. Chen, A. W. Thomas, C. Catania, N. D. Kirchhofer, L. E. Garner, A. Han and G. C. Bazan, *Adv. Mat.*, 2013, **25**, 1593-1597.
14. A. W. Thomas, L. E. Garner, K. P. Nevin, T. L. Woodard, A. E. Franks, D. R. Lovley, J. J. Sumner, C. J. Sund and G. C. Bazan, *Energ. Environ. Sci.*, 2013, **6**, 1761-1765.
15. V. B. Wang, J. Du, X. Chen, A. W. Thomas, N. D. Kirchhofer, L. E. Garner, M. T. Maw, W. H. Poh, J. Hinks, S. Wuertz, S. Kjelleberg, Q. Zhang, J. S. C. Loo and G. C. Bazan, *Phys. Chem. Chem. Phys.*, 2013, **15**, 5867-5872.
16. J. Du, A. W. Thomas, X. Chen, L. E. Garner, C. A. Vandenberg and G. C. Bazan, *Chem. Commun.*, 2013, **49**, 9624-9626.
17. C. K. Ng, K. Sivakumar, X. Liu, M. Madhaiyan, L. Ji, L. Yang, C. Tang, H. Song, S. Kjelleberg and B. Cao, *Biotechnol. Bioeng.*, 2013, **110**, 1831-1837.
18. X. Li, H. Xu, Z.-S. Chen and G. Chen, *J. Nanomater.*, 2011, **2011**, 16.
19. P. Sharma, M. J. Teixeira De Mattos, K. J. Hellingwerf and M. Bekker, *FEBS Journal*, 2012, **279**, 3364-3373.
20. J. K. Fredrickson, 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, *Nat. Rev. Microbiol.*, 2008, **6**, 592-603.
21. S. Orsetti, C. Laskov and S. B. Haderlein, *Environ. Sci. Technol.*, 2013, **47**, 14161-14168.
22. T. Zhang, C. Cui, S. Chen, H. Yang and P. Shen, *Electrochem. Commun.*, 2008, **10**, 293-297.
23. Y. Qiao, C. M. Li, Z. Lu, H. Ling, A. Kang and M. W. Chang, *Chem. Commun.*, 2009, 6183-6185.
24. P. S. Guin, S. Das and P. C. Mandal, *Int. J. of Electrochem.*, 2011, **2011**.
25. R. Gulaboski, I. Bogeski, V. Mirceski, S. Saul, B. Pasieka, H. H. Haeri, M. Stefova, J. P. Stanoeva, S. Mitrev, M. Hoth and R. Kappl, *Sci. Rep.*, 2013, **3**.
26. M. Bekker, G. Kramer, A. F. Hartog, M. J. Wagner, C. G. de Koster, K. J. Hellingwerf and M. J. de Mattos, *Microbiology (Reading, England)*, 2007, **153**, 1974-1980.
27. F. J. M. Hoeben, P. Jonkheijm, E. W. Meijer and A. P. H. J. Schenning, *Chem. Rev.*, 2005, **105**, 1491-1546.
28. K. Mitra, I. Ubarretxena-Belandia, T. Taguchi, G. Warren and D. M. Engelman, *Proc. Natl. Acad. Sci.*, 2004, **101**, 4083-4088.

