Bacteria to Control Electron Transport Altering the Synthesis of Non-native Biopolymer

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The use of bacteria as catalysts for radical polymerisations of synthetic monomers has recently been established. However, the role that transplasma membrane electron transport (tPMET) plays in modulating these processes is not well understood. We sort to study this by genetic engineering of a part of a tPMET system NapC in E coli and show this altered the rate of extracellular electron transfer and caused an effect on cell-mediated polymerisation using a model monomer. A plasmid with arabinose inducible PBAD promoters were shown to upregulate NapC protein upon arabinose induction at total arabinose concentrations of 0.0018% and 0.18%. These clones (E. coli (IP_0.0018%) and E. coli (IP_0.18%), respectively) were used in Fe atom transfer radical polymerization (ATRP), revealing faster polymerization rates than cultures containing suppressed or empty plasmids (E. coli (IP_S) and E. coli(E), respectively). These results lead to the hypothesis that EET (Extracellular Electron Transfer) mechanisms in part modulate rates of ATRP iron-mediated cell instructed polymerizations.

Introduction

Electron transfer plays a fundamental role in modulating biology, enabling almost all cellular functions.[1] Transplasma membrane electron transport systems [tPMETs] are essential from a biological energetic perspective and for mediation of cell redox and signaling.[2, 3] It has recently been shown that electron transfer via tPMETs can be used to synthesise biopolymers and may have a role in radical polymerisations of synthetic monomers.[4-8] This was achieved by tPMET modulating the redox state of metal ions facilitating atom transfer radical polymerisation or redox-mediated oxidative polymerisation events. Importantly, the fabrication of *in-situ* generated biopolymers opens new strategies for interfacing cells with new materials and may find applications controlling cell behaviour and connecting cellular bioelectrical relays.[9, 10] To date however, the underlying cellualr systems that modulate polymerisation have not been studied in depth. One area that is expected to moudlate the synthesis of polymers is bacterial electron transfer, however, there is a lack of understanding of how biologically derived electron transfer affects polymer synthesis.

One approach to controlling bacterial electron transfer is to adopt synthetic biology protocols to tune bacterial behaviour.[11] Consequently, we were inspired to explore a synthetic biology approach to tune electron transfer from bacteria for iron (Fe) mediated atom transfer radical polymerisation (ATRP) under aqueous conditions. It has been previously shown that electrogenic *Cupriavidus metallidurans (C. met)*[12-15] and *Escherichia coli (E. coli)* are able to start Fe ATRP reactions.[4] Metal reduction takes place via microbial external electron transfer (EET) involving membrane bound C type Cytochrome (C-Cyt) proteins, possibly in combination with synergistic Fe homeostasis efflux pumps.[9] To further understand the role of microbial EET in Fe catalysis and resulting polymerisation, C-Cyt (NapC of *E. coli*) was our engineering target. C-Cyt was chosen as a target to mediate cell instructed ATRP as the cytochromes are a class of enzymes that contribute to bacterial EET to produce energy, particularly involving the reduction of metals.[16-19] The electron transport chain of *S. oneidensis MR1 i*s well researched and the corresponding C-Cyts can contribute to metal reduction,[20, 21] with protein CymA integral to the reduction of iron.[22]

NapC is also a member of the NapC/NirT family of C-Cyt, existing in the periplasmic membrane of *E. coli*.[23] NapC showed ferric reductase activity like its homologue, CymA, with the ability to substitute for CymA when inserted into *Shewanella* strains.[24] As *E. coli* K12 has been previously shown to initiate ATRP with Cu-,[7] and Fe-catalysts,[4] it was sought as a vehicle to investigate the effect of EET on polymerisation by altering NapC levels. To study the resulting change in EET we used an electrochemical method we have recently reported.[2, 25] Herein we find that overexpression of the NapC protein in *E. coli* alters EET and this changes the kinetics of the reported cell instructed bio-mediated polymerisations.

We initially took a synthetic biology approach to rewire tPMET behaviour. We began by cloning ***napC*** gene isolated from *E. coli* into the plasmid pMTL83153 (details are given in **Table S1**), immediately downstream from the non-constitutive inducible promoter (P_{BAD}). We chose P_{BAD} because high quantities of transcribed protein (NapC) were desired to facilitate investigations into its effect on iron reduction which is indicative of EET. P_{BAD} would also allow us to control any potential toxicity and NapC expression by optimising the concentration of inducer (arabinose). To achieve this, a pMTL8000 modular plasmid collection was designed for ease of component selection during cloning. This can also be useful for tuning via plasmid replicons (controls replication efficiency), markers (antibiotic resistance selection), promoters (drives the transcription of the target gene), and multiple cloning sites (MCSs) (containing restriction sites for restriction enzyme cloning).**[26]** Although these were created to aid cloning in Clostridium cultures, they are hosted in *E. coli* and so were convenient in the cloning of *napC* into *E. coli*. The plasmid constructed, based on pMTL83153 **(Figure 1a)**, confers resistance to chloramphenicol **[27]**[26]and *napC* under the control of PBAD.

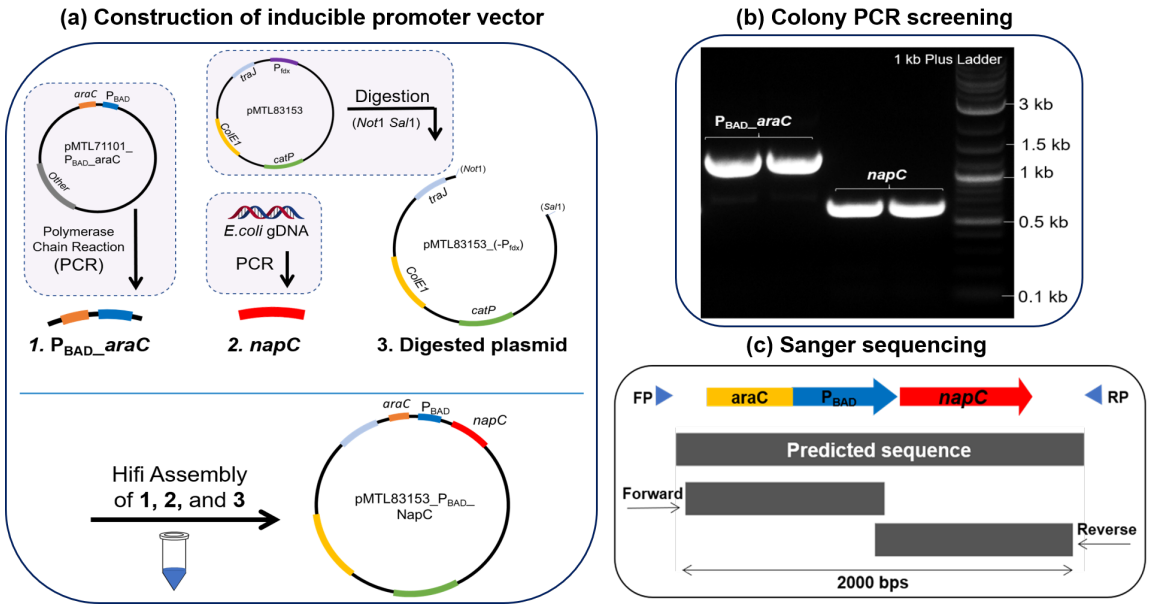

Figure 1. Assembly of Inducible Promoter Vector containing promoter P_{BAD} for NapC overexpression and control. (a) PCR was carried out with specific primers to extract and amplify regions 1, P_{BAD}_araC and 2, napC. Region 1 was obtained from the plasmid pMTL71101_P_{BAD}_araC and region 2 was obtained from *E. coli* gDNA. The plasmid pMTL83153 was digested with REs *Not*I and *Sal*I to remove the Pfdx promoter, resulting in region 3, digested plasmid. The three regions were ligated together with Hifi Assembly to create the completed vector. (b) Gel electrophoresis of Colony PCR products for DNA regions making up the Inducible promoter vector, against 1 KB Plus DNA ladder P_{BAD}_araC DNA region (1278 bps) and napC DNA region (633 bps) are as expected. (c) Sanger sequencing diagrams for Inducible promoter vector showing matching DNA regions of sequencing with forward and reverse primers compared to a model sequence.

Plasmid pMTL83153 was assembled using the *napC* gene and P_{BAD} promoter region, amplified from *E. coli* and plasmid pMTL71101_P_{BAD}_araC, respectively, amplified and purified. These *napC* and P_{BAD} regions were inserted in *Not* and *Sal*I digested pMTL83153. The transformation of the cloned vectors led to the growth of colonies that were selected on Cm plates and screened by Colony PCR (Primers: ColE1+tra_F2 and pCB102_R1; Table S2). Gel electrophoresis was used to analyse the resultant amplicons and confirm the successful

insertion of P_{BAD}_araC DNA region (1278 bps) and *napC* DNA region (633 bps) (Figure 1b). Finally, the authenticity of the cloned pMTL83153_ P_{BAD}_NapC inducible promoter vector colonies (*E. coli*$_{(IP)}$) was confirmed using Sanger sequencing of the amplified DNA fragments (Figure 1c) and designated pBADNAP.

After the successful cloning of *napC* , we examined the resulting NapC protein expression by *E. coli*. NapC expression was studied alongside clones harbouring a control plasmid, pMTL83151, referred to as the 'Empty Plasmid' (*E. coli*$_{(E)}$) . NapC expression in bacteria containing pBADNAP (*E.coli*$_{(BADNAP)}$) was induced by the addition of arabinose, which binds to AraC, activating the P_{BAD} promoter and initiates transcription of the protein. This expression was tuned by optimising the arabinose concentration **(Table S3)** and its effect on NapC content was examined by performing SDS-PAGE (**Figure S1**). *E. coli*$_{(BADNAP)}$ were exposed to a final arabinose concentration of 0% (band I_0), 0.000018% (band I_1), 0.0018% (band I_2), and 0.18% (band I_3). As a control measure, arabinose was also added to *E. coli*$_{(E)}$ (band E), permitting a consistent comparison between protein expression levels. The total protein quantification was analysed using bicinchoninic acid (BCA) protein assay. **(Figure S2) (Table S4)**. A certain band of interest was identified in the resulting protein gel **(Figure S1, circled green area)**.) **(Figure S3)**.[23] An increase in the intensity of bands was observed at higher arabinose inductions (I_2 and I_3), and vice versa with no induction (I_0) or *E. coli*$_{(E)}$ (Band E). The intensity of bands I_0 and I_1 show similar intensities but an increase was observed for I_2 and I_3 suggesting that a certain amount of arabinose induction (0.0018%) is necessary for P_{BAD} actively to promote NapC overexpression. A further increase in arabinose concentration (0.18%) only increases the intensity slightly indicating a limit of expression.

As shown previously, Fe ATRP can be activated by bacteria including *E. coli*.[4] To investigate the effects of NapC overexpression on Fe reduction rates, *E. coli* $_{(BADNAP)}$ were used in Fe ATRP reactions **(Table S5)** alongside *E. coli*$_{(E)}$. An iron catalysed ATRP of the water-soluble monomer poly(ethylene glycol) methyl ether methacrylate (PEGMA, Mn = 300 gmol@1) was carried out at 37°C with tris(2-

dimethylaminoethyl)amine (Me_6TREN) for Fe^{3+} reduction. The kinetics of each reaction was monitored by ^{1}H NMR **(Figure 2a)** and the resulting polymers were analysed by SEC **(Figure S4 and Table 1)**. An increased rate of polymerisation was observed by the larger polymer yield for *E. coli*$_{(BADNAP\_0.0018\%)}$ and *E.coli*$_{(BADNAP\_0.18\%)}$ activated reactions, compared to those activated by *E. coli*$_{(E)}$ or *E. coli*$_{(BADNAP\_S)}$ cultures. This suggested that the upregulation of the NapC protein had some effect on the rates of Fe^{3+} reduction to Fe^{2+}. As this difference is small, it suggests that there could be other factors contributing to EET from the bacteria to the Fe catalyst. These might include (electron transfer) ET across the periplasm via other cascade proteins in the electron transport chain, or EET between shuttle molecules and the Fe catalyst (**Figure 2b**)**.**

To note the Đ[c] values suggest the polymerisation is not very well controlled. However, we do show bioelectrical activity is one factor effecting the mechanism of polymerisation and will be discussed in more detail in the proceeding experiments. Howvere, more research is required to deconstruct the other biological controlling factors.

Table 1. Fe ATRP polymerisations activated by *E. coli* cultures harbouring different plasmids to compare the effects of NapC protein upregulation.

Culture	**Conversion[a]**	**M_n^{th} (kDa)[b]**	**M_n^{SEC} (kDa)[c]**	**Đ[c]**
E. coli$_{(E)}$	38.4%	23.3	251.9	3
E. coli$_{(IP_S)}$	38.8%	23.5	237.4	2.9
E. coli$_{(IP_0.0018\%)}$	46.5%	28.1	228	2.7
E. coli$_{(IP_0.18\%)}$	45.6%	27.6	230	3

We next wanted to study the effect on electron transfer when altering the NapC expression levels of the tPMET. This was achieved using linear sweep voltammetry (LSV), an electrochemical technique that reports on the concentration of ferricyanide and ferrocyanide (Fe reduction), which is indicative of the electron transfer rate when measured over time. We have previously reported this LSV based method to detect concentration changes corresponding

to EET in cells.[25, 28, 29] The first derivative function of the LS voltammograms **(Figure S5)** obtained from known concentrations of ferricyanide/ferrocyanide redox couple was used to calculate the rate of change in steady-state current ($d(I_{ss})/dt$) **(Figure 3a)**. A calibration curve of concentration vs ($d(I_{ss})/dt$) **(Figure S6)** was generated to determine whether *E. coli*$_{(IP)}$ clones with upregulated NapC protein could reduce more Fe^{3+} in the form of ferricyanide. Next, the samples of *E. coli*; *E. coli*$_{(E)}$, *E. coli*$_{(IP\_S)}$ (suppressed by addition of glucose to inhibit P_{BAD} activation of NapC expression),[30] *E. coli*$_{(IP\_0.0018\%)}$, and *E. coli*$_{(IP\_0.18\%)}$ were incubated with ferricyanide for 1 hour at 37 °C, after which the bacteria were removed by centrifugation and the supernatant was taken and LSV was performed on the supernatant. The average current was taken for each sample and the first derivative graph was plotted to determine $d(I_{ss})/dt$ values. Finally, the $d(I_{ss})/dt$ values of each sample were compared to the calibration graph and the concentration of ferrocyanide (Fe^{2+}) in solution was plotted as a percentage of total Fe concentration.

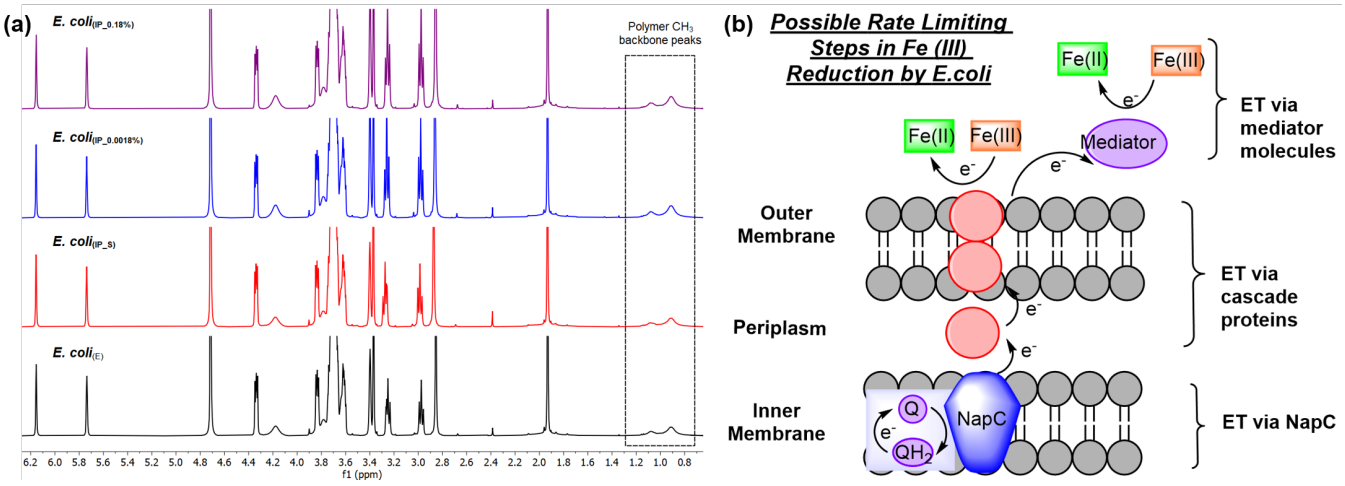

Figure 2. Fe ATRP polymerisations activated by *E. coli* cultures harbouring different plasmids to compare the effects of NapC protein upregulation. (a) ^{1}H NMR spectra of Fe ATRP activated by E. coli(E) (black), or inducible promoter plasmids, E. coli$_{(IP)}$ either (i) suppressed by addition of glucose E. coli$_{(IP_S)}$ (red), ii) activated by 0.0018% total arabinose concentration E. coli$_{(IP_0.0018\%)}$ (blue) or ii) activated by 0.18% total arabinose concentration E. coli$_{(IP_0.18\%)}$ (purple). (b) Possible Rate limiting steps in Fe(III) reduction including electron transfer (ET) via NapC, ET via cascade proteins and ET via mediator molecules.

The data indicate that *E. coli*$_{(E)}$ and *E. coli*$_{(IP\_S)}$ cultures reduced more ferricyanide to ferrocyanide than the *E. coli*$_{(IP)}$ clones **(Figure 3b)**. This is of note because higher ferricyanide reduction was expected from *E. coli* clones that were induced with arabinose as they were shown to overexpress NapC. We attribute this to a shift in the bioenergetics of the bacteria, that could have been triggered in *E. coli*$_{(IP\_0.0018\%)}$ and *E. coli*$_{(IP\_0.18\%)}$ cultures, whereby EET reduction pathways were stunted due to the use of excess energy required for the over-production of the NapC protein. Interestingly, there was a slight increase in the ferricyanide reduction with *E. coli*$_{(IP\_0.18\%)}$ cultures compared to *E. coli*$_{(IP\_0.0018\%)}$ cultures. Previous MIC studies revealed that increasing total arabinose concentrations, to 0.18% induced detrimental effects on the metabolism of *E. coli*$_{(IP)}$ (**Figure S7**). This increased stress in *E. coli*$_{(IP\_0.18\%)}$ cultures could have triggered EET systems (such as NapC) increasing ferricyanide reduction. Stress induced tPMET upregulation pathways were also observed in analogous experiments with other cell types,[2] suggesting that cells utilise EET systems to balance bioenergetic requirements. Although the bioenergetics of Fe metabolism in living organisms remains challenging to study,[31] further studies are underway to gain more insights through monitoring Fe reducing behaviour in stress-induced environments, such as temperature, pH and chemical treatments.

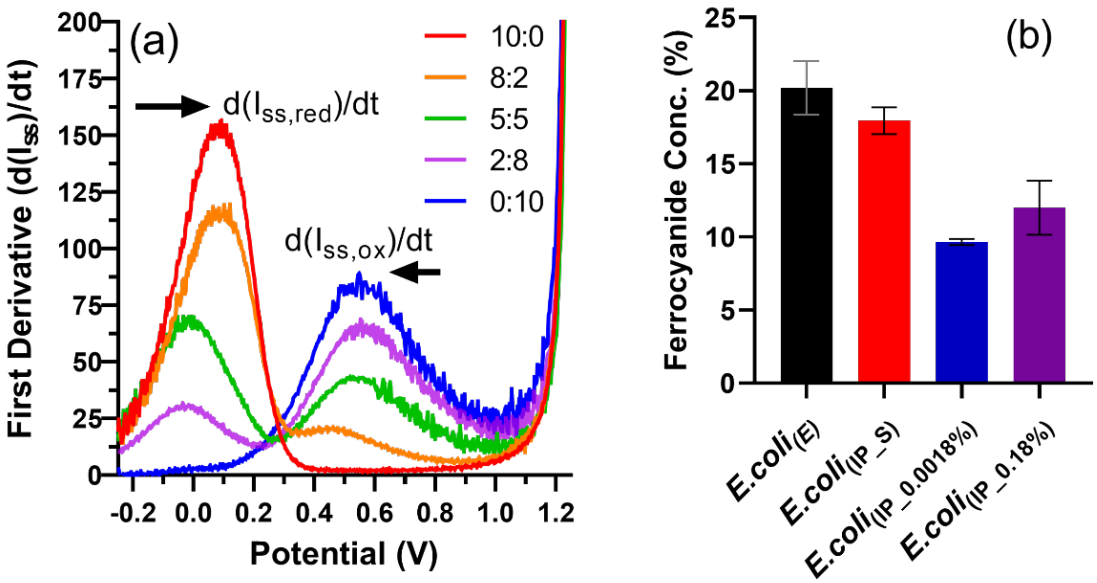

Figure 3. Electrochemical detection of ferrocyanide using Linear sweep voltammetry. (a) First derivative function applied to Linear sweep voltammogram of ferricyanide/ferrocyanide redox couple (Fig S6) to determine d(I_{ss})/dt values. (b) Concentrations (%) of ferrocyanide detected in the supernatant of samples incubated for 1 hour with *E. coli*$_{(IP)}$ or E. coli$_{(E)}$. LSV was used to analyse the supernatant of incubated samples (N=2, n=6) and the first derivative function was applied to resulting voltammograms. The concentrations were determined using the calibration graph (Figure S7).

Conclusions

In summary, the cloning of the *napC* gene into *E. coli* for subsequent overexpression of NapC protein was achieved and controlled with the P_{BAD} promoter. We established that there were differences in electron transfer rates. The polymerisation rates for ATRP catalysed by *E. coli*$_{(IP\_0.0018\%)}$ and *E. coli*$_{(IP\_0.0018\%)}$ were faster, suggesting that NapC upregulation has some effect on the Fe^{3+} reduction system. This subsequently affects the nature of the polymerisation. On the modification of NapC levels, there appear to be alterations in extracellular electron transfer which coincides with variations in differences in polymerisation kinetics.

Acknowledgments

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Author Contributions

Conceptualization: M.R.B., F.J.R., C.A. P.J.H, **Data curation:** M.R.B., F.J.R., C.A. and K.K. **Formal Analysis:** M.R.B., F.J.R., and K.K. **Funding acquisition:** F.J.R. **Investigation:** M.R.B. **Methodology:** M.R.B., F.J.R. and C.A. **Project administration:** F.J.R. **Resources:** F.J.R., CA, P.J.H, K.K. **Supervision:** C.A., P.J.H., F.J.R. **Validation: M.R.B., A.J., C.A., F.J.R. Visualization:** F.J.R., M.R.B and C.A. **Writing –** A.J., M.R.B. and F.J.R. **Writing – review & editing:** A.J., M.R.B., F.J.R., CA, P.J.H, K.K.

Conflicts of interest

There are no conflicts to declare.

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