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Membrane permeabilization by conjugated oligoelectrolytes accelerates whole-cell catalysis†

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Conjugated oligoelectrolytes (COEs) boost the electrical performance of a wide range of bioelectrochemical systems, yet their mechanism of action remains incompletely understood. One possible mode of action is that COEs permeabilize the cell envelope. We thus examined the effect of tetracationic COE, DSSN+, on the permeability of the inner and outer membrane of *Escherichia coli* by detecting extracellular activity of normally periplasmic and cytoplasmic enzymes. DSSN+ increases the release of the periplasmic enzyme alkaline phosphatase (ALP) up to 20-fold, but does not significantly change the release of the cytoplasmic enzyme β-galactosidase. Additionally, DSSN+ caused a 2-fold increase in the turnover of a cytoplasmic substrate. These studies present a more complete understanding of the mechanism of action in bioelectrochemical systems and pivot future applications of COEs towards a method for improving whole-cell catalysis.

Introduction

The cell envelope acts as a protective barrier that limits the transport of ions, non-natural sugars and redox-active molecules in the cell.[1–5] In Gram-negative bacteria, the envelope is comprised of an outer membrane (OM), a peptidoglycan layer and an inner membrane (IM). In the OM, lipopolysaccharides provide a barrier to hydrophobic and most hydrophilic molecules. Small hydrophilic compounds (<600 Da) rely on passive diffusion through OM porins, while large hydrophilic molecules typically require specific protein-based transport mechanisms.[1,2,6] In contrast, transport of hydrophobic molecules through the IM is relatively facile, while the transport of hydrophilic molecules typically requires specific membrane transport proteins.[7,8] For reactants that do not have a natural uptake system, passive diffusion through the lipopolysaccharide layer is the only mechanism of transport.[9]

The inherent barrier function of the OM can be a limitation in whole-cell bioprocesses such as bioelectrochemistry, biocatalysis, fermentation and bioremediation.[10] In an ideal process, uptake of substrates into the cell would not slow the rate of product formation. In practice, however, whole-cell catalyzed reactions are generally one to two orders of magnitude slower than catalysis by isolated enzymes.[8,11] Chemical and physical methods have been developed to increase membrane permeability and accelerate reaction rates despite possible complications in downstream processing, added processing steps, and excessive membrane damage or lysis.[8,12] While genetic modifications can also improve permeability, they are often more effective at enhancing transport across the IM than the OM and are complicated to implement alongside other genetic modifications, such as enzyme overexpression.[13] Thus, there is a paucity of well-characterized, one-step methods for altering membrane permeability that do not introduce additional limitations.

Cationic conjugated oligoelectrolytes (COEs) such as 1,4-bis(4′-(*N*,*N*-bis(6″-(*N*,*N*,*N*-trimethylammonium)hexyl)amino)-styryl)benzene tetraiodide, referred to as DSSN+ (Fig. 1a), are of particular interest for their ability to modify membrane properties and improve performance of bioelectrochemical devices.[14,15] COEs have a π-delocalized backbone bearing ionic pendant groups and are known to spontaneously intercalate into lipid bilayers. COEs have shown to improve electrochemical performance in devices utilizing yeast,[13] *Escherichia coli*,[16] *Shewanella oneidensis*,[10,15] mixed communities,[17] and even membrane-based photosystems.[18–20] A major reason DSSN+ has been so widely used is because of its relative biocompatibility, with a minimum inhibitory concentration to growth of 256 μM in *E. coli*.[10,15,21,22]

Despite its utility, the origin of DSSN+ improvement in bioelectrochemical devices remains under debate.[9,10,17,21,23–30] Model membrane studies have shown that COEs increase ion conductance across the membrane.[13] To add to mechanistic complexity, recent results suggest that COEs may interact with other components in the cell envelope, such as lipopolysaccharides and cholic acid.[31–33] The degree to which such

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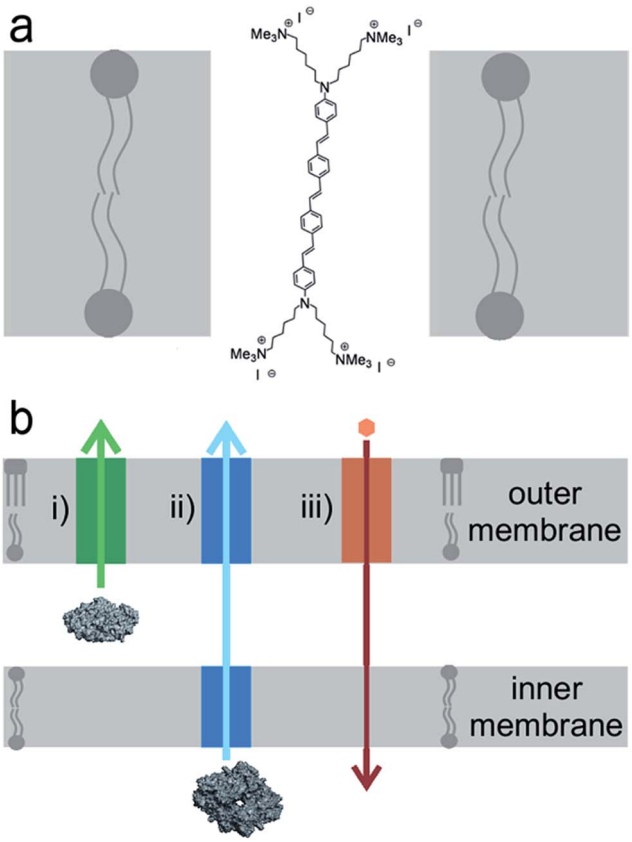

Fig. 1 Schematic illustration depicting DSSN+ situated in the lipid bilayer (a) and the scenarios tested in this study (b). DSSN+ intercalation in the membrane may either: (i) permeabilize the OM to allow the release of periplasmic enzymes, (ii) permeabilize the IM to release cytosolic enzymes, and/or (iii) increase the rate of catalysis by intracellular enzymes by increasing the rate of transport of substrate across the cell envelope.

processes dominate bioelectrochemical changes, relative to electron transfer mechanisms, is not entirely known. Regardless, it is generally acknowledged that the ability of COEs to spontaneously intercalate in the membrane underlies their ability to increase charge transfer in bioelectrochemical devices.[33]

Given the importance of membrane permeabilization on bioprocesses, a better understanding of how COEs alter membranes would open opportunities beyond bioelectrochemical applications. Here, we address possible impacts on membrane permeabilization and whole cell catalysis by using the approach illustrated in Fig. 1. Specifically, we examine changes in the OM and IM permeability of *E. coli* upon exposure by detecting extracellular enzymatic activity of normally periplasmic (i) and cytosolic (ii) enzymes. We find that the permeability of the OM is significantly affected by DSSN+ (scenario (i)) as indicated by extracellular enzyme activity, while we do not yet find evidence for perturbation of the IM (scenario (ii)). Furthermore, by monitoring turnover of the substrate utilized by a cytosolic enzyme (scenario (iii)), we demonstrate DSSN+ increases whole-cell biocatalysis.

Results and discussion

Outer membrane permeability assay

A standard protocol was used to test changes in OM permeability (scenario (i) in Fig. 1b). We measured the extracellular activity of the periplasmic enzyme alkaline phosphatase (ALP) after *E. coli* was exposed to different concentrations of DSSN+ for varying lengths of time. Specifically, *E. coli* cells were washed, resuspended in buffer, then stained with 5, 10, 15 and 25 μM DSSN+ for 1, 4 or 10 hours then filtered. The resulting filtrates were probed for extracellular ALP activity by monitoring conversion of non-fluorescent 4-methylumbelliferyl phosphate (MUP) to fluorescent 4-methylumbelliferone (4-MU).[30,34,35] The fluorescence intensity linearly increased over 60 min for filtrates from both unstained, untreated (UT) and DSSN+ treated cells (selected examples are shown in Fig. 2). However, the rate of fluorescence increase was greater in filtrates from DSSN+ treated cells relative to UT cells (Fig. 2). Thus, DSSN+ alters the OM permeability sufficiently to facilitate periplasmic release of ALP, which is a moderately-sized macromolecule of 89 kDa.[36–38]

To quantitatively assess the degree of OM permeabilization under different conditions, we calibrated the ALP activity of different percentages of *E. coli* cell lysate. As with the filtrates of DSSN+ stained and UT samples, the 4-MU fluorescence of the cell lysates linearly increased with time (*e.g.* 50% cell lysate solution, Fig. 2). A linear trend was generated between the rate of fluorescence change and amount of cell lysate, thus providing a calibration curve between extracellular ALP activity and the relative degree of OM lysis, described as a percentage of total intracellular ALP (see ESI† for detailed methods). We use this calibration curve to determine the relative degree of cell lysis, notated by $L_{OM\%}$, in which $L_{OM\%}$ values of 100 and

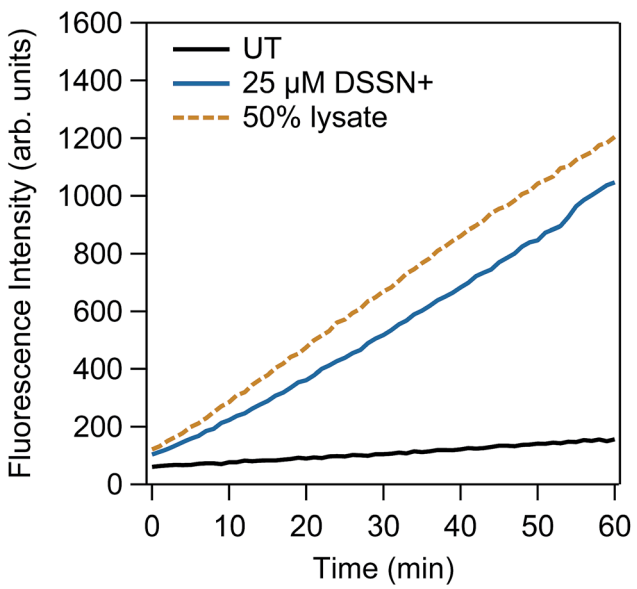

Fig. 2 Extracellular ALP activity of filtrates from UT (solid black), *E. coli* stained with 25 μM DSSN+ for 10 hours (solid blue), and a 50% cell lysate solution (dashed yellow), determined by fluorescence intensity of 4-MU measured at 440 nm over time. All measurements are an average of triplicates.

0 correspond to a fully lysed and fully intact cell population, respectively.

We compared the effective OM lysis of *E. coli* cells as a function of DSSN+ staining concentration and time (Fig. 3). As summarized in Table 1 and plotted in Fig. 3, the $L_{OM\%}$ values increased with both DSSN+ staining concentration and time. Specifically, *E. coli* cells stained with the greatest DSSN+ concentration (25 μM) showed the most pronounced increase in OM permeability. The OM permeability of these cells was 20-fold higher than UT cells at 4 h, and at 10 h had the greatest $L_{OM\%}$, *i.e.* 45%. Given that the DSSN+ exposure in these experiments do not cause measurable cytotoxicity,[10,21,22] these data indicate DSSN+ is able to increase OM permeability without significantly altering *E. coli* cell viability. These observations demonstrate that scenario (i), namely enzyme transport across the OM, is accelerated for cells that are treated with DSSN+. It is worth noting that freeze/thaw methods used to release similar concentrations of recombinant proteins are poorly effective for proteins of ALP's dimensions.[39]

From Fig. 3, one observes that changes in OM permeability increase as the cells are exposed to more DSSN+; however, the magnitude of this increase tapers with both DSSN+ staining concentration and time. At concentrations over 10 μM, the effect of additional DSSN+ on OM permeability is diminished. It is worth pointing out that the maximum association of DSSN+ with *E. coli*, previously shown to be 20 nmol/$OD_{600\ nm}$, is equivalent to a staining concentration of 12 μM in our experimental conditions.[32] These data thus suggest that little additional DSSN+ associates with cells above 10 μM, causing only a minor additional impact on OM permeability. Additionally, the OM permeability increases rapidly over the first 4 h of staining, then tapers for all DSSN+ concentrations. These data indicate that DSSN+ association with cells reaches a steady-state after 4 h of staining. Taken together, these data are consistent with the suggestion that the degree of OM permeability is related to the amount of DSSN+ associated with the cell and that DSSN+ association with *E. coli* cells saturates at a staining concentration of ~10 μM and a staining time of ~4 h.

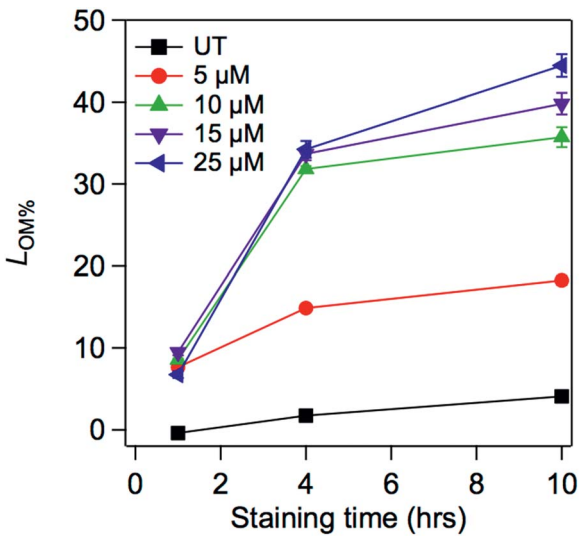

Fig. 3 Permeabilization of the OM of *E. coli* from varying concentrations of DSSN+ measured as a percentage of total ALP activity ($L_{OM\%}$) released extracellularly over staining time. Error bars show standard deviation of an average of triplicates.

Table 1 Effect of DSSN+ staining on OM permeability at 4 h, IM permeability at 5 h, and the rate of ONPG hydrolysis, defined as the linear rate of absorbance increase

[DSSN+] (μM)	$L_{OM\%}$	$L_{IM\%}$	Turnover rate (a.u. min^{-1})
0 (UT)	1.7 ± 0.12	0.8 ± 0.002	0.009 ± 0.001
5	14.9 ± 0.27	1.0 ± 0.003	0.013 ± 0.003
10	31.8 ± 0.36	1.2 ± 0.006	0.018 ± 0.007
15	33.7 ± 0.79	1.3 ± 0.005	0.018 ± 0.007
25	34.2 ± 1.01	1.3 ± 0.005	0.018 ± 0.004

Inner membrane permeability assay

To determine whether DSSN+ had a similar impact on IM permeability (scenario (ii) in Fig. 1b), we measured the release of the cytoplasmic hydrolase enzyme, β-galactosidase (β-gal), into the medium, which is commonly used as an indicator of inner membrane damage.[40–44] As before, *E. coli* cells were washed in buffer, stained with varying concentrations of DSSN+ for 5 hours and then filtered. Extracellular β-gal activity in the resulting filtrates was measured by monitoring cleavage of colorless 2-nitrophenyl β-D-galactopyranoside (ONPG) into yellow *o*-nitrophenol (ONP) by using absorption spectroscopy. For filtrates from both UT cells and cells stained with DSSN+,

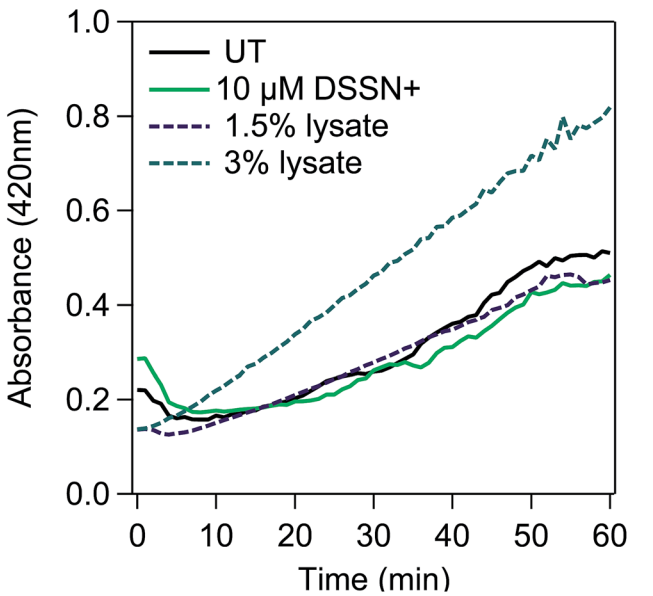

Fig. 4 Permeation of *E. coli* IM by DSSN+ determined optically by measuring absorbance of ONP at 420 nm. Extracellular β-galactosidase activity in the filtrates from UT (solid black) and 10 μM DSSN+ stained *E. coli* (solid green) shown over incubation time with ONPG and compared with absorbance of 1.5% (dashed purple) and 3% (dashed blue) cell lysate solutions. All measurements are an average of triplicates.

the ONP absorbance at 420 nm linearly increased with time (Fig. 4). In contrast to the ALP experiments, there was no marked difference in the rate of change of $A_{420\ nm}$ between UT and DSSN+-stained cell filtrates, even when [DSSN+] was increased up to 25 μM. This observation indicates that IM permeability for β-gal did not change significantly upon DSSN+ staining and therefore scenario (ii) is not operational under our experimental conditions.

To more precisely quantify the effects of DSSN+ staining on IM permeability, we assessed the ONP production rate for different amounts of IM lysis and calculated $L_{IM\%}$ for each staining concentration (Table 1). The ONP formation rate for 3% cell lysate was significantly higher than both the UT and 10 μM DSSN+ stained cell filtrates (Fig. 4), while the rate for 1.5% lysate was comparable to these samples. These data show that the amount of β-gal released by DSSN+ staining is both comparable to unstained cells and very small. Taken together, these data indicate that the IM is not sufficiently disrupted to accelerate β-gal diffusion out of the cell. This is an important improvement to our current understanding of how DSSN+ improves charge transport in *E. coli*, which was previously suggested to be caused by cell lysis.[9]

When considering the difference DSSN+ has on OM and IM permeability, it is important to consider that the OM and IM permeabilities were probed with different size proteins. With a molecular weight of 465 kDa, β-gal is ~5 times larger, than ALP (MW = 89 kDa).[36–38,45,46] While the mode by which DSSN+ increases membrane permeability is unclear, it is possible that these changes in permeability may be size dependent. Regardless, as release of β-gal is often used as evidence of cell lysis, it is clear that DSSN+ does not cause *E. coli* lysis under the experimental conditions studied here.[9,41,43,44,47]

Whole-cell hydrolysis

Considering the ability of DSSN+ to increase the OM permeability, we hypothesized DSSN+ could improve the transport of much smaller substrate molecules through the cell envelope (scenario (iii) in Fig. 1b). Because ONPG hydrolysis is rate limited by its passive diffusion through the OM, we chose to test this hypothesis using a modified β-gal assay on whole *E. coli* cells stained with DSSN+.[43,48–50] Cells were therefore incubated with varying concentrations of DSSN+ for 5 hours, washed, and then monitored for ONP production as a function of time. The time dependence of the background-corrected $A_{420\ nm}$ *versus* time provides a measure of product formation (Fig. 5). The faster increase in $A_{420\ nm}$ for DSSN+ stained cells shows that DSSN+ stained cells produce ONP more rapidly than unstained counterparts.

To quantify the relative ONP production rate, we determined the slope of the linear region in $A_{420\ nm}$ *versus* time plots shown in Fig. 5. As observed with the ALP experiments, this increase in turnover rate increased with DSSN+ staining concentration up to ~10 μM. This limiting value reinforces the idea that permeability changes are proportional the amount of DSSN+ associated with the cell.[32] A comparison of these turnover rates (Table 1) shows that DSSN+ stained cells hydrolyzed ONPG up to 2-fold more rapidly than unstained cells. This 2-fold increase in turnover does not match with the 20-fold increase in OM permeabilization, indicating a different rate limiting process may be occurring. Regardless, while DSSN+ staining does not release β-gal from the cytoplasm, it does increase transport of ONPG across the cell envelope, resulting in increased microbial catalysis.

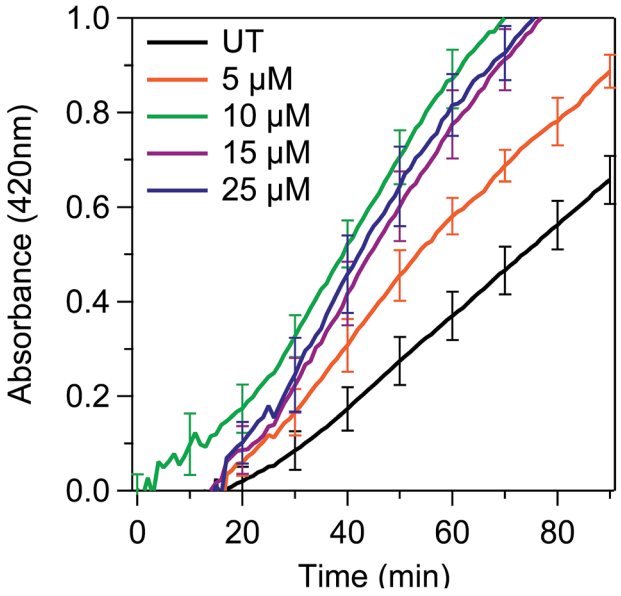

Fig. 5 Turnover of ONPG by whole cells monitored by absorbance of ONP at 420 nm over time. Error bars are standard deviations of triplicates and plotted for every 10 minutes.

While it is possible that DSSN+ may affect transport of molecules or proteins with a low molecular weight across the IM, at this point we do not have definitive proof that DSSN+ is permeabilizing or, to what extent, even reaching the IM. Regardless, this interest in structural elucidation has led to the discovery of a new way to accelerate whole-cell catalysis by improved transport across the OM. Beyond whole-cell catalysis, these observations have broader implications where membrane permeabilization is relevant, for example antimicrobial susceptibility.[7,8]

The data presented here also offers mechanistic insight into how DSSN+ increases current production in *E. coli* microbial fuel cells (MFCs).[21,27] In contrast to previous reports, the IM remains primarily intact under our experimental conditions.[9] Since the conditions described herein closely mimic that used in MFCs, it is unlikely that the increased extracellular electron transfer (EET) in MFCs is due to cell lysis. Rather, given the substantial increase in OM permeability with DSSN+ staining, we suggest DSSN+ increases transport of redox active molecules or electron donors in the media across the OM.[21,27] Increased transport of either set of molecules would increase the observed current in an *E. coli* MFC. Supporting this idea, increasing the permeability of the OM in *E. coli via* directed evolution,[51–53] overexpression of porins,[50,54] and introduction of permeabilizers[55] have all been previously used to increase mediated EET. Additionally, a recent study demonstrated that released enzymes in certain microorganisms

can mimic direct EET.[56] It is also important to note that COEs alter the physiochemical properties of the cell in a manner that may affect cell–electrode interactions.[32] Thus we cannot rule out that DSSN+ improves *E. coli* MFC performance through a combination of effects, such as increased permeability and cell attachment.

While the specific mechanism of membrane perturbation is yet to be defined experimentally, molecular dynamic simulations demonstrated distortions and disorder in the phospholipid bilayer upon COE modification and aggregation of COEs.[10] To what extent the LPS layer is modified is less well understood. It is not unreasonable to suggest COE interactions with LPS cause disorder, which is a common cause of OM permeability.[8]

Experimental

Cell culture

Escherichia coli K-12 (ATTC #10798, ATCC, VA) was cultured aerobically overnight from a frozen stock by growth in Luria Broth (10 g L^{-1} bacto tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl) at 37 °C with shaking. For cells cultured for β-galactosidase activity assays, Luria Broth was supplemented with 2% lactose for induction of *lacZ*. Cells were washed three times by centrifugation in M9 minimal media (6.8 g L^{-1} Na_2HPO_4, 3 g L^{-1} KH_2PO_4, 1 g L^{-1} NH_4Cl, 0.5 g L^{-1} NaCl) before use in assays.

Assay of outer membrane permeation

Cell suspensions were resuspended to a final $OD_{600\ nm} = 1.0$ in M9, stained with varying amounts of 1 mM DSSN+ in ultrapure water to achieve final concentrations of 0, 5, 10, 15 and 25 μM, and then incubated at room temperature for 1, 4 and 10 hours. Samples were removed at each time point and filtered using a 0.2 μm filter. Non-fluorescent 4-methylumbelliferyl phosphate disodium salt (MUP) from an alkaline phosphatase activity assay kit (Biovision, K422-500) was used to measure the activity of extracellular ALP. Filtered samples were incubated with 80 μM MUP at 25 °C in a 96-well plate while monitoring fluorescence (Ex/Em = 360 nm/440 nm) over 90 min using a Spectra Max Plus 384 microplate spectrophotometer. Filtrates without MUP added were measured for background correction. All samples were measured in triplicate.

Assay of inner membrane permeation

Cell suspensions were resuspended to a final $OD_{600\ nm} = 0.6$ in M9, stained with varying concentrations of DSSN+ (0, 5, 10, 15 and 25 μM) and then incubated at room temperature for 5 hours. The permeability of the inner membrane of *E. coli* K-12 was determined by measuring the release of β-galactosidase activity into the medium. Cell suspensions were filtered, and the resulting filtrates were incubated with 1.3 mM 2-nitrophenyl β-D-galactopyranoside (ONPG, Sigma Aldrich) at 37 °C for 2 hours in a 96-well plate while monitoring the absorbance at 420 nm using a Spectra Max Absorbance plate reader. All samples were measured in triplicate.

Calibration against cell lysates

For both permeability assays, activities were compared against the activity of lysed cell suspensions. Cell suspensions of the same $OD_{600\ nm}$ and same culture conditions used in the comparative assays were lysed *via* probe sonication in an ice bath. Complete lysis was confirmed by measuring $OD_{600\ nm}$ to be equal to 0. Lysates were then serially diluted in M9 to produce dilutions of 50, 25, 12.5, 6.1, 3 and 1.5% cell lysate. These solutions were then tested following the respective assay procedures.

Substrate turnover assay

Cell suspensions were resuspended to an $OD_{600\ nm} = 0.6$ in M9, stained with varying concentrations of DSSN+ (0, 5, 10, 15 and 25 μM) for 30 minutes to allow for membrane intercalation, then centrifuged and resuspended in M9 to a final $OD_{600\ nm} = 0.6$. Cell turnover of ONPG, indicated by the linear rate of absorbance increase, was examined in triplicate for each staining concentration using a Spectra Max Absorbance plate reader to measure absorbance at 420 nm over 2 hours while cells were incubated with 1.3 mM ONPG. Background corrections were made by subtracting the absorbance at 420 nm of DSSN+ stained cell suspensions that were not incubated with ONPG.

Conclusions

In summary, we have shown that DSSN+ increases the permeability of the OM and improves transport of a small molecule through the cell envelope. By measuring the release of the periplasmic ALP enzyme into the extracellular medium, it is possible to unify changes in the OM permeability with accumulation of DSSN+. This process led to a ~20-fold increase in OM permeability. This effect was observed in staining concentrations far below those needed for toxicity. We did not observe significant release of β-gal, indicating DSSN+ does not adequately disturb the IM to allow outward diffusion of this enzyme. This does not rule out accumulation of DSSN+ in the IM since the dimensions of the protein may be too large for diffusion. It may be possible that smaller macromolecules could diffuse, but this requires additional tests. Nonetheless, it is important to note these results have led to the discovery of a new way to accelerate whole-cell catalysis. Last, we show that DSSN+ staining increases the rate of ONP formation up to 2-fold.

The deeper understanding of DSSN+'s impact on OM permeability suggests COEs may be suited for wide range of whole-cell applications.[57,58] Here, we have shown DSSN+ improves passive transport across the OM. Thus, COEs provide a synthetic, one-step method to either release a periplasmic product or increase transport of substrate across the OM by using specifically designed synthetic molecules with limited influence on cell viability. Thereby, use of COEs for increased transport across the OM has the potential to improve microbial catalysis, recombinant protein recovery, or even decrease antibiotic resistance, where the OM is limiting.

Acknowledgements

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