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Development of a novel fluorescence probe capable of assessing the cytoplasmic entry of siderophore-based conjugates

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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A novel fluorescence probe capable of assessing the cytoplasmic entry of siderophore-based conjugates was synthesized and evaluated by photochemical characterization and cell-based assays. The specific responsiveness to the cytoplasmic entry of the probe was implemented by adopting a disulfide linker whose cleavage under reducing conditions of the cytoplasm induced display of a distinctive fluorescence signal.

Siderophores are high affinity iron chelators produced and utilized by most bacteria as the primary means to assimilate the iron from the aerobic environment, in which the solubility of Fe(III) is extremely low.[1,2] Because of the essentiality of the iron for many biological functions including oxygen storage, energy transfer, and catalysis, the mechanisms related to the siderophore utilization such as biosynthesis, secretion, and active uptake have been considered as promising novel antibiotic targets.[3] One of the actively pursued approaches is to utilize the siderophore as the drug delivery vector by constructing siderophore-drug conjugates (SDCs).[4-7] This design, inspired by natural product antibiotics such as salmycins and albomycins, has been anticipated to be effective in treating Gram-negative pathogens, as their drug resistance is often associated with the restricted membrane permeability.[8,9]

Indeed, many artificial SDCs have been prepared and tested for their antibiotic activity against various Gram-negative bacteria. Interestingly, increased or comparable activity compared to the parent antibiotic drug has been observed mostly when the SDCs feature drugs functioning on periplasmic targets such as penicillin-binding proteins (PBPs).[10-13] The conjugation with quinolone antibiotics targeting DNA gyrase in the cytoplasm has been found ineffective to Gram-negative bacteria, suggesting that the attachment of a siderophore might attenuate the activity of the parent drug[14] or the corresponding SDCs might have limited access to the cytoplasm.[12,15] Most siderophores except for pyoverdines are known to deliver the iron intracellularly by penetration into the cytoplasm through designated uptake systems composed of TonB-dependent outer membrane receptors, periplasmic binding proteins, and ATP-dependent inner membrane permease complexes.[16] Thus, if conjugation of drug molecules to the siderophore has detrimental effects on the interactions of siderophores with uptake machineries located at the periplasm and/or the inner membrane, the cytoplasmic entry of SDCs can be inhibited.

The restricted access of SDCs only to the periplasm can be problematic in finding potent SDC antibiotics, because the repertoire of drugs to be conjugated becomes significantly limited. Nonetheless, there has been no systematic study to address this issue until very recently, in which Nolan and co-workers have found that the size of conjugated molecules is an important parameter to affect the transport of enterobactin-based conjugates into the cytoplasm of *Escherichia coli* and *Pseudomonas aeruginosa*.[14] In this regard, we herein report development of a novel fluorescence siderophore probe responsive to the cytoplasmic entry, thus allowing detailed analysis of the trafficking of siderophore-based conjugates. Specifically, the design concept, preparation of a model probe **6**, photochemical characterization, as well as cell-based evaluation are described.

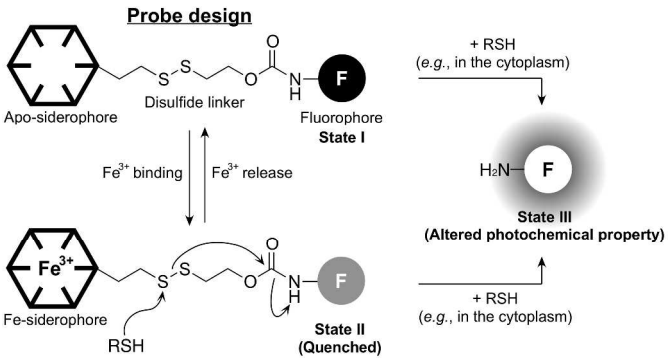
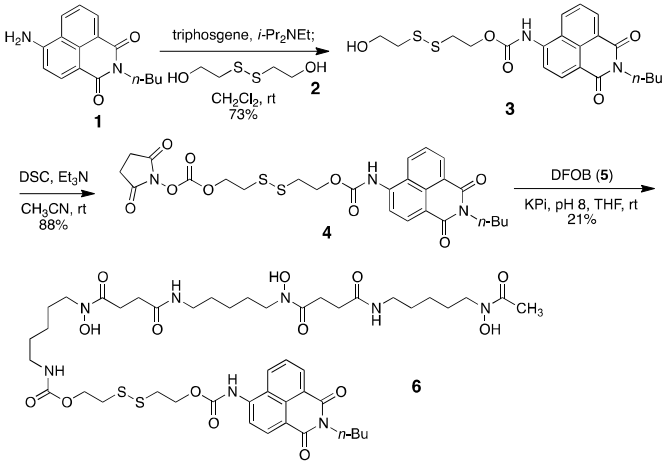


Fig. 1 The concept of the new probe design.

The concept of our new probe design is depicted in Fig. 1. Essentially, its structural organization composed of a fluorophore, a siderophore, and a linker is similar to previously known siderophore-fluorophore conjugates,[17-21] but distinctive by the presence of a disulfide bond on the linker. In bacteria such as *E. coli*, the cytoplasm is maintained under reducing conditions by free thiols such as the thioredoxins and glutathione whereas the periplasm is kept in an oxidative state primarily by a disulfide-forming enzyme DsbA.[22] Accordingly, our probe can be specifically responsive upon the entry into the cytoplasm, where cellular thiols cleave the disulfide bond triggering the liberation of a free amine form of the fluorophore with altered photochemical property (State I or II to State III, Fig. 1). This disulfide cleavage-based strategy has been exploited in development of a number of intracellular thiol probes and drug delivery systems particularly in mammalian cells.[23-25] In addition, the new probe is also capable of detecting the reversible Fe(III) binding as other siderophore-fluorophore conjugates, because this event often accompanies the fluorescence quenching (State I to State II, Fig. 1). Thus, our novel probe design allows simultaneous monitoring of two separate behaviours of a siderophore-based conjugate, chelation to Fe(III) as well as cytoplasmic entry.



Scheme 1 Synthesis of a model probe **6**.

As a model probe, we decided to synthesize compound **6** featuring 4-amino-1,8-naphthalimide (**1**) and desferrioxamine B (DFOB, **5**) as the fluorophore and the siderophore, respectively, that are connected via a disulfide linker **2**. The synthesis of **6** relied on successive carbamate formations on the both free hydroxy ends of **2** with fluorophore **1** and DFOB (**5**) as described in Scheme 1. Briefly, the connection between **1** and **2** was effected by treatment of triphosgene to afford **3**. The second carbamate linkage was elaborated first by priming the free hydroxyl group as the NHS anhydride. Subsequent application of DFOB (**5**) to the activated intermediate **4** in a buffered solution then successfully yielded probe **6** after purification by reverse-phase HPLC.

With probe **6** in hand, spectroscopic characterization was conducted to validate the probe design *in vitro*. Specifically, the changes in the absorption and fluorescence profiles upon exposure to glutathione (GSH), a representative cellular free thiol, or Fe(III) or both were monitored. As shown in Fig. 2A, treatment of GSH to **6** induced redshift (from 370 nm to 440 nm) in absorption, consistent with the liberation of **1** (Fig. S2). The fluorescence also underwent changes, in which probe **6** alone displayed strong emission (max at ca. 470 nm) upon excitation at 370 nm, whereas only marginal emission was observed from the sample treated with GSH (Fig. 2B). In contrast, the excitation at 440 nm gave rise to strong emission (max at ca. 540 nm) on the GSH-treated sample in contrast to **6**

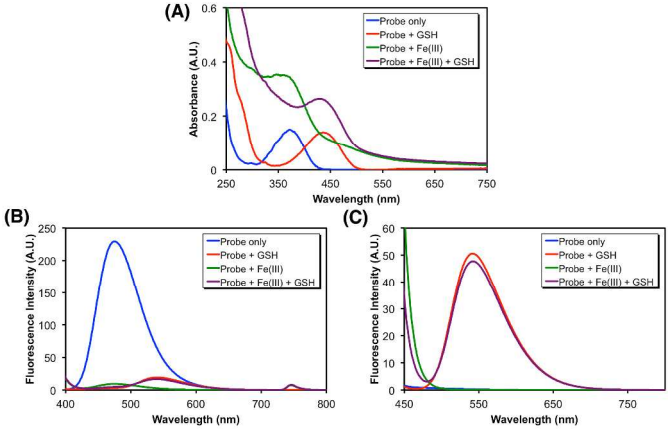


Fig. 2 Spectroscopic characterization of **6** (10 μM, blue) under different conditions selectively involving glutathione (5 mM, red) or $FeCl_3$ (100 μM, green) or both (violet) at 37 ºC in 100 mM Tris•HCl (pH 8, 50% methanol). (A) UV/vis absorption spectra, (B) fluorescence spectra upon the excitation at 370 nm, (C) at 440 nm.

alone showing no response (Fig. 2C). Then, the effect of the iron binding was tested. While no apparent change in the position of the absorption maximum was observed upon exposure to $FeCl_3$ (Fig. 2A and S1), the Fe(III) chelation to **6** was found to effectively quench the fluorescence emission at 370 nm excitation as anticipated (Fig. 2B). The cleavage of the disulfide bond by GSH appeared not affected by association with Fe(III) (Fig. 2C). Collectively, these spectral data support that probe **6** is differentially, albeit not fully independently, responsive to the Fe(III)-binding and the cytoplasmic entry as designed, of which events can be readily assessed by monitoring two separate fluorescence channels, excitation near 370 nm and 440 nm, respectively.

To validate the function of probe **6** in the cell-based settings, two Gram-negative strains, *Pseudomonas putida* ATCC 12633 and *E. coli* BW25113, were selected. Although they do not produce DFOB endogenously, they are capable of utilize it as a xenosiderophore.[26,27] *E. coli* BW25113, a K-12 derivative, depends on FhuCDB for the inner membrane transport of DFOB, but this strain lacks a high affinity outer membrane receptor for DFOB. Thus, we transformed *E. coli* with pFU2 containing the *foxA* gene, an outer membrane ferrioxamine receptor from *Yersinia enterolitica*, to increase the DFOB uptake capacity.[28]

Each strain was cultured in an iron-deficient succinate medium up to the mid-exponential phase, and Fe(III)-**6** (5 μM) was added after the adjustment of OD_{600} to 1. After 1.5 hr incubation at 37 ºC, the fluorescence emissions were monitored in a 96-well microplate reader. As shown in Fig. 3A and 3B, incubation of Fe(III)-**6** with *P. putida* exhibited discernible fluorescence increases upon excitation at both 355 nm and 460 nm, which correspond to the Fe(III) release from Fe(III)-**6** and liberation of fluorophore **1** via cleavage of the disulfide bond in the probe, respectively. Treatment of NaN_3, a known inhibitor for energy-dependent processes, significantly decreased the emissions by 39% and 31% at 355 nm and 460 nm excitation, respectively. In addition, co-treatment of ferrioxamine B (FOB) also led to slight fluorescence decreases at 355 nm and 460 nm excitations, both by 13%. These antagonizing effects by NaN_3 and FOB collectively support that the observed fluorescence changes are driven by the actions of DFOB-specific uptake machineries. Fluorescence increase at 460 nm excitation requiring the presence of free thiols is particularly noteworthy as it indicates that Fe(III)-**6** is capable of penetrating inside the cytoplasm of *P. putida*. The fluorescence increase at 355 nm excitation suggests that dissociation of Fe(III) from the ferrioxamine scaffold in Fe(III)-**6** would precede

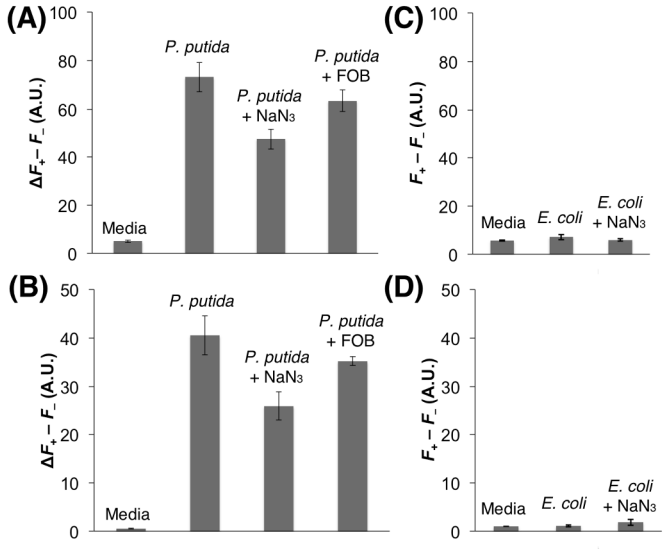


Fig. 3 Evaluation of the cellular uptake of Fe(III)-**6** in *Pseudomonas putida* [(A), (B)] and *Escherichia coli* [(C), (D)]. The differences in the fluorescence signals between the presence and the absence of the probe, Fe(III)-**6**, ($F_+ - F_-$) are presented. (A), (C) Emission at 460 nm upon excitation at 355 nm. (B), (D) Emission at 535 nm upon excitation at 460 nm. The test condition is indicated on the top of each column. All experiments were performed at least in triplicates and the error bars indicate the standard deviations.

the disulfide cleavage upon its delivery to the cytoplasm and some portions of iron-free **6** would remain intact. In contrast, incubation of Fe(III)-**6** with *E. coli* did not elicit any fluorescence change either upon the excitation at 355 nm or at 460 nm (Fig. 3C and 3D). The possibility of insufficient cellular thiol contents in the *E. coli* cytoplasm or slow kinetics of the thiol-promoted disulfide cleavage could be ruled out because treatment of Fe(III)-**6** to the *E. coli* lysates displayed strong fluorescence emission at 460 nm excitation (Fig. S5). These results suggest that the *E. coli* strain used herein is incapable of accepting Fe(III)-**6**, indicative of the adverse effects of the fluorophore appendage in Fe(III)-**6** on the interactions with DFOB-uptake machineries in *E. coli*. The difference in the transport capacity for Fe(III)-**6** between *P. putida* and *E. coli* is intriguing, and it suggests that the DFOB-specific uptake machineries in these two organisms would have different ligand specificity. While the sequence alignments could provide some insights into this observation (see the Supplementary Information, Fig. S9), a more rigorous answer would require extensive structural analysis and binding studies.

Conclusions

Herein, we presented a novel fluorescence probe design capable of assessing the cytoplasmic entry of siderophore-based conjugates. The model probe based on DFOB, Fe(III)-**6**, was observed to be accessible into the cytoplasm of *P. putida*, but not of *E. coli*. These contrasting results clearly demonstrate that our probe design can be readily utilized to compare the cytoplasmic entry of siderophore-based conjugates among different bacteria. Our probe design is distinguished from other methods commonly used to investigate the cellular uptake of siderophores, based on the use of radioactive Fe(III)[29] or conventional siderophore-fluorophore conjugates,[17-21,30] in that its response is directly related to the cellular location of the probe itself, whereas others rather correspond to the trajectory of Fe(III), not that of the siderophore. Thus, our new probe design would allow more detailed tracing of the cellular uptake pathway of siderophore-based conjugates, particularly cytoplasmic entry thereof. This disulfide linker-based design is readily adaptable to other siderophores and thus can be used for direct comparison of the cytoplasmic uptake efficiency of various different siderophore-based conjugates, of which information should have significant contributions in discovery of new potent SDC antibiotics.

Acknowledgements

This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF-2012R1A1A1042665, NRF20100020209) and TJ Science Fellowship of POSCO TJ Park Foundation. We would also like to thank Prof. Klaus Hantke for providing us pFU2 plasmid.

Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental procedures, spectral data for all new compounds, copies of spectra, and results of photochemical characterization and cell-based assays. See DOI: 10.1039/c000000x/

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