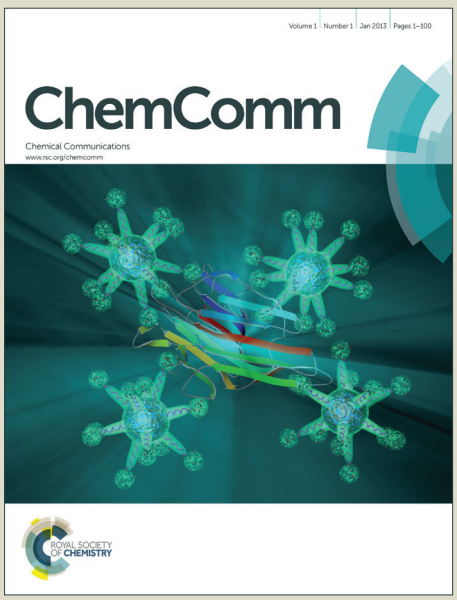
ChemComm

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

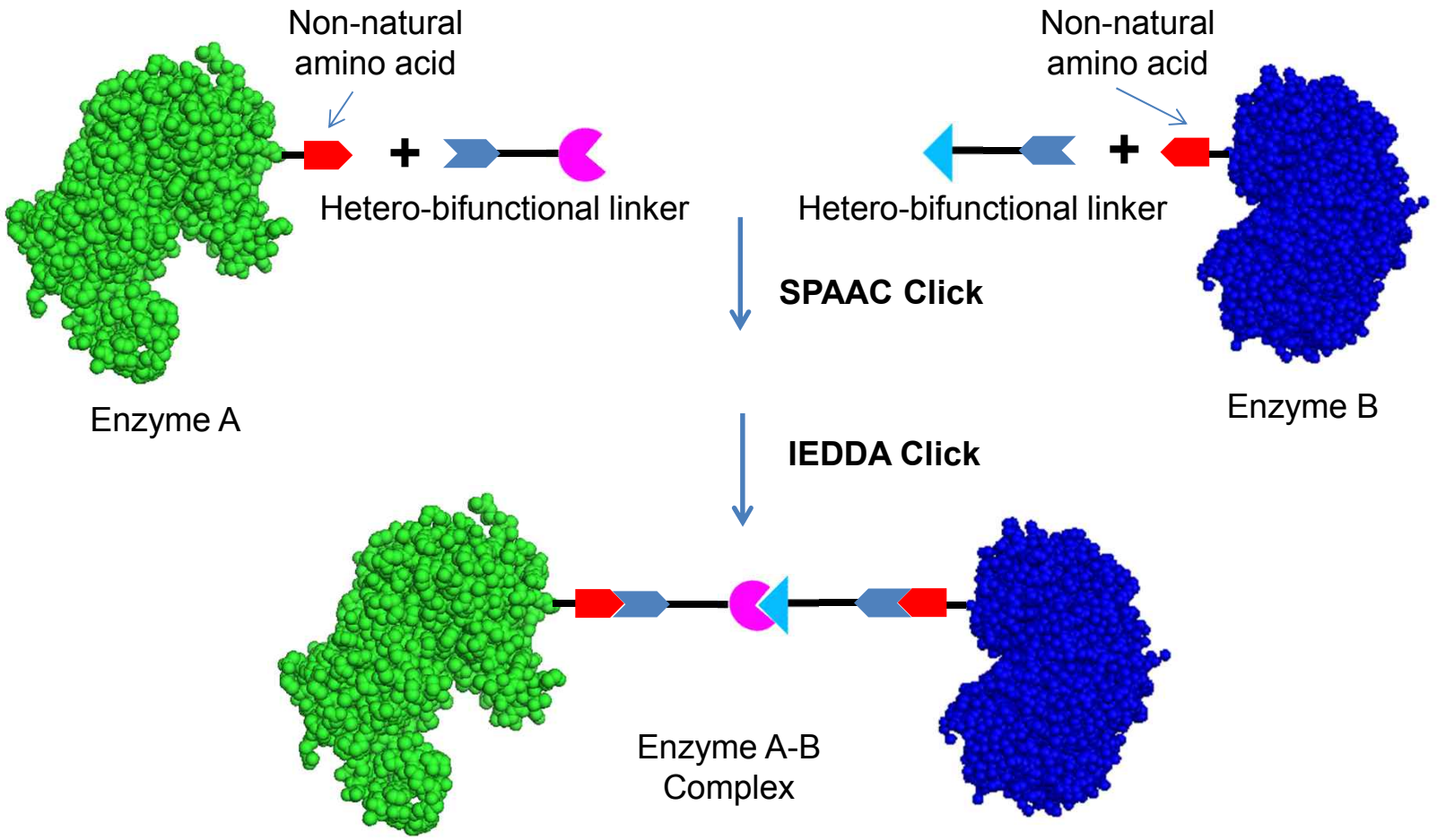
You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Here, we report a novel strategy to site-specifically couple multiple enzymes using two compatible click chemistries and site-specific incorporation of a clickable non-natural amino acid.



ChemComm Accepted Manuscript

ChemComm

COMMUNICATION

Double clicking for site-specific coupling of multiple enzymes

Sung In Lim,[a] Jinhwan Cho,[b] and Inchan Kwon*[a,b]

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

A method to site-specifically couple multiple enzymes is reported. The approach is based on site-specific incorporation of a clickable non-natural amino acid into enzymes and two compatible click reactions. The multi-enzyme reaction system generated exhibited the enhanced catalytic efficiency over the respective free enzymes.

In nature, multiple enzymes in one metabolic pathway often form enzyme complexes to efficiently catalyze cascade reactions via intermediate channelling effect. Similarly, covalent coupling of multiple enzymes is considered a very promising strategy to enhance the enzymatic reaction efficiency for production of value-added chemicals.[1-3] Although genetic fusion and covalent coupling of enzymes at amines or thiol groups were utilized to construct multiple-enzyme complexes,[4-9] these techniques have poor control over the coupling site and crosslinking process. Coupling at multiple sites inevitably generate a mixture of enzyme conjugates with varying compositions.[10] Furthermore, conjugation to the enzyme active site most likely compromises the catalytic activities. In order to enhance the control over the coupling site and enzyme-complex configuration, several elegant approaches were developed such as co-immobilization on a DNA scaffold, residue-specific incorporation of a non-natural amino acid, and enzyme-mediated conjugation.[1, 11-13] However, there was still some restriction in choosing coupling sites.

In this study, we designed a strategy to achieve an efficient, convenient conjugation of multiple enzymes with an absolute site-specificity using two compatible click reactions as well as site-specific incorporation of a clickable non-natural amino acid (Fig. 1A and 1B). First, a clickable *p*-azido-L-phenylalanine (AZF) was introduced into two enzymes (enzymes A and B) with the retained catalytic activity. Then, two clickable hetero-bifunctional linkers (HBL-1 and HBL-2) were conjugated to two different enzymes via the first click reaction strain-promoted azide-alkyne cycloaddition (SPAAC), respectively. Finally, two enzyme-linker conjugates were coupled via the second click reaction, inverse electron-demand Diels-Alder reaction (IEDDA), to generate a multi-enzyme reaction system (Fig. 1B).

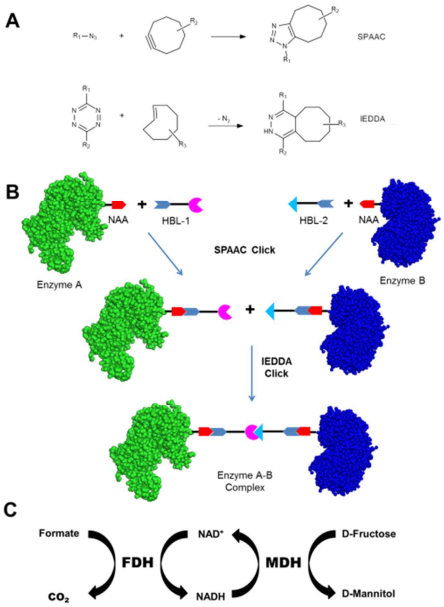


Fig. 1 Schematics showing the click reactions, construction of a multi-enzyme system, and coupled enzyme reactions. (A) Two bioorthogonal click chemistries—strain promoted azide-alkyne cycloaddition (SPAAC) and inverse electron-demand Diels-Alder reaction (IEDDA). (B) A strategy to construct a multi-enzyme reaction system by conjugating two enzymes via consecutive click reactions (SPAAC and IEDDA). NAA, Non-natural amino acid containing an azido group; HBL-1, Hetero-bifunctional linker containing dibenzocyclooctyne (DBCO) and tetrazine; HBL-2, Hetero-bifunctional linker containing DBCO and *trans*-cyclooctene (TCO) groups. (C) The enzymatic D-mannitol synthetic reaction coupled with cofactor regeneration catalyzed by formate dehydrogenase (FDH) and mannitol dehydrogenase (MDH).

Recently, site-specific conjugation of enzymes receives great attraction, because enzyme conjugation can be made at permissive sites. Once a clickable non-natural amino acid was introduced into

[a] Department of Chemical Engineering, University of Virginia, 102 Engineers' Way, Charlottesville, VA 22904, United States.
[b] School of Materials Science and Engineering, Gwangju Institute of Science and Technology (GIST), 123 Cheomdan Gwagi-ro, Buk-gu, Gwangju, 500-712, Republic of Korea. E-mail: inchan@gist.ac.kr; Fax: +82-62-715-2304; Tel: +82-62-715-2312

† Electronic Supplementary Information (ESI) available: Experimental details of preparation of plasmids, *E. coli* expression cells, and recombinant enzymes, conjugates, and enzymatic assays. See DOI: 10.1039/x0xx00000x

ChemComm Accepted Manuscript

enzymes, a click reaction, such as copper-catalyzed azide-alkyne cycloaddition (CuAAC) or SPAAC, was employed to immobilize enzymes onto solid surface.[14-17] When a conjugation site was carefully chosen, the enzyme retained the catalytic activity even after the conjugation.[18] Bundy et al. previously reported that two clickable non-natural amino acids, *p*-propargyloxy-L-phenylalanine and *p*-azido-L-phenylalanine, were introduced into two proteins, respectively, for direct protein-to-protein conjugation via CuAAC.[19] Despite its simplicity and site-specificity of coupling, the application of this technique to enzymes seems limited due to a potential issue of activity loss caused by copper ions.

In this study, site-specific coupling of multiple enzymes were achieved via two consecutive click reactions in order to achieve the absolute site-specificity in coupling site and retained enzyme activity (Fig. 1B). As a model system, we chose a pair of formate dehydrogenase (FDH) and mannitol dehydrogenase (MDH). FDH is a homodimer with a molecular mass of 45 kDa for a single subunit originating from *Thiobacillus* sp. KNK65MA, and catalyzes the conversion of formate into carbon dioxide by reducing cofactor NAD^+ into NADH.[20, 21] MDH, derived from *Pseudomonas fluorescens*, is a monomer with a molecular mass of 55 kDa, and catalyzes the reduction of D-fructose to D-mannitol by consuming NADH.[22, 23] In the cascade reactions consisting of FDH and MDH (Fig. 1C), NADH is regenerated by FDH-cataltyzed formate oxidation, thereby continuously fueling MDH-catalyzed D-mannitol production. In the presence of excess substrates, formate and D-fructose, for both enzymes, the transfer of NADH between the active sites of FDH and MDH governs the overall cascade reaction efficiency.

As a first step to construct FDH-MDH conjugates, conjugation sites were carefully chosen. Several factors were taken into consideration. First, in order to avoid the activity loss upon coupling, coupling sites should not be involved in a native function. Second, in order to minimize the structural perturbation upon incorporation of AZF, residues with a hydrophobic side chain, such as phenylalanine, tryptophan, and valine, were chosen as strong candidates. Third, in order to achieve an efficient coupling, residues with a relatively high solvent accessibility were selected. Solvent accessibility of residues ranging from 0 (no accessibility) to 1 (full accessibility) was evaluated by ASA-View web-based program.[24] According to our previous results, residues with the ASA value greater than 0.4 were suitable as a conjugation site.[14, 18, 25, 26] Based on these criteria, the valine at position 237 of FDH (0.423 ASA index) and the valine at position 417 of MDH (0.462 ASA index) were determined as coupling sites (Fig. 2A).

Site-specific genetic incorporation of AZF was performed by introduction of an amber codon into predetermined sites of FDH- and MDH-encoding genes. Host cells were induced to express an orthogonal pair of amber suppressor tRNA and tRNA synthetase as well as the target gene in the presence of AZF in culture medium. The expression yield of AZF-bearing FDH and MDH was 5 and 8 mg/L, respectively. Purified FDH and MDH variants were analyzed by dye labeling and mass spectrometry to verify the bioorthogonal reactivity and site-specific incorporation of AZF, respectively. In contrast to the wild-type FDH (FDH-WT) and MDH (MDH-WT) showing no fluorescence when mixed with a DBCO-functionalized fluorescence dye, variants exhibited a strong fluorescence (Fig. S1). MALDI-TOF mass spectra of tryptic fragments demonstrated the high fidelity incorporation of AZF in response to the amber codon at position 237 for FDH and position 417 for MDH, respectively (Fig. 2B). To investigate the effect of AZF incorporation on the native activity, variants were subjected to the enzymatic activity assay in comparison to the wild type enzymes (Fig. 4A). Both variants containing AZF (FDH-AZF and MDH-AZF) retained enzymatic activities comparable to that of their respective wild-type.

Since genetically encoded AZFs have been found to be situated at chemically well-defined positions and have bioorthogonal reactivity towards SPAAC, variants of FDH and MDH provide a modular platform to generate FDH-MDH conjugates, through a chemical linker. To cross-link FDH-AZF to MDH-AZF, FDH-AZF was reacted with a DBCO-tetrazine linker (Fig. 3A) through SPAAC to generate FDH-TET, and desalted to remove residual linkers. Likewise, MDH-AZF was conjugated to a DBCO-PEG12-TCO linker to generate MDH-TCO (Fig. 3A). The majority of catalytic activity of each enzyme was retained even upon the linker conjugation (Fig. 4A). A second bioorthogonal reaction, IEDDA, was carried out to covalently link FDH-TET to MDH-TCO to generate the FDH-MDH conjugate (Fig. 3A). In SDS-PAGE analysis of the reaction mixture, a single band slightly larger than the 200 kDa-standard protein was detected, indicating the FDH-TET reacted with MDH-TCO to form FDH-MDH (Fig. S2). The slower migration than expected from the total molecular weight of the monomeric FDH-MDH conjugate, 110 kDa, resulted from a long and flexible PEG spacer which retarded mobility through the gel matrix. The FDH-MDH conjugate was isolated from the reaction mixture by performing an anion exchange chromatography. Since the FDH dimer became dissociated in SDS-PAGE, two discrete bands were resolved with the upper band corresponding to the monomeric FDH conjugated to MDH while the lower band showing the same molecular weight with the unmodified monomeric FDH (Fig. S2). An apparent size increase upon FDH-MDH conjugation was confirmed in comparison to dimeric FDH and MDH by size-exclusion

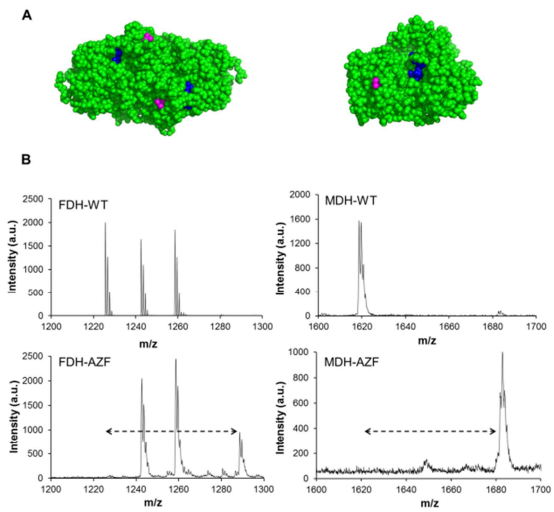


Fig. 2 Genetic incorporation of AZF and validation of its site-specificity. (A, left) A dimeric form of FDH in complex with a cofactor (blue) was derived from Protein Data Bank (PDB ID: 3WR5). The AZF incorporation site, V237, is highlighted in magenta. (A, right) MDH in complex with a cofactor (blue) was derived from Protein Data Bank (PDB ID: 1LJ8). AZF incorporation site, V417, is highlighted in magenta. (B) MALDI-TOF MS analyses of trypsin-digested FDH-WT, MDH-WT, and their variants.

ChemComm Accepted Manuscript

 This journal is © The Royal Society of Chemistry 20xx

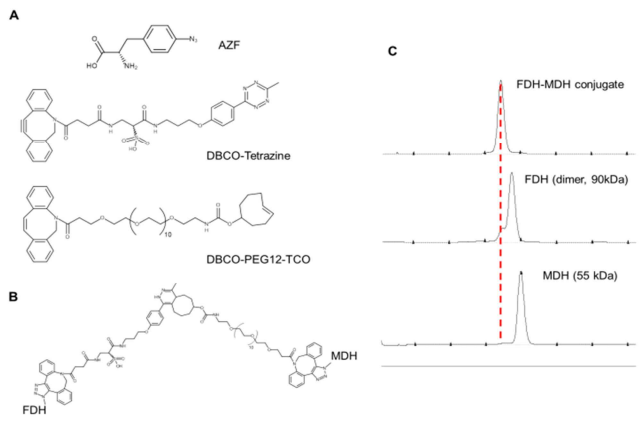


Fig. 3 Synthesis and size characterization of the FDH-MDH conjugate. (A) Chemical formulae of AZF and DBCO-derivatized bifunctional linkers. The DBCO group reacted with AZF incorporated into FDH and MDH via SPAAC. The conjugation between FDH and MDH was mediated by IEDDA between tetrazine and TCO. (B) The entire structure of the conjugate focused on the formula for the chemical linker bridging FDH and MDH. (C) The elution profile of the conjugate by size-exclusion chromatography in comparison to unmodified FDH-WT and MDH-WT.

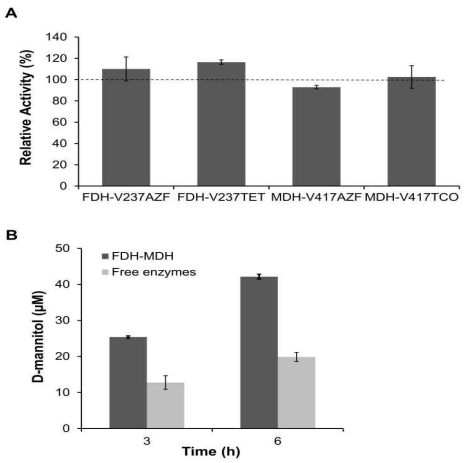


Fig. 4 Enzymatic activity assays. (A) Catalytic activities of FDH and MDH variants plotted in comparison to those of their respective wild-type (100%). (B) Catal... performance of the FDH-MDH conjugate relative to free enzymes in the multi-enzyme reaction system for D-mannitol production. The FDH-MDH conjugate ... the basis of 5 nM MDH) or a comparable amount of unconjugated FDH and MDH (5.5 nM dimeric FDH and 5 nM MDH) was subjected to the cofactor-regenerating cascade reaction in the presence of 500 μM NAD^+ and 50 mM of formate and ... fructose, respectively. Concentrations of the product, D-mannitol, were measured at 3 and 6 hrs after the reaction began.

chromatography (Fig. 3C). The conjugate exhibited a sharp and symmetric peak with an elution time earlier than its parents without any detectable impurity, indicating its high homogeneity and purity. The reaction yield at each step of the conjugate synthesis is summarized in Table S1.

Due to dimeric nature of FDH, however, the FDH-MDH conjugate may display two different configurations, i.e. a single MDH attached to either subunits of a dimeric FDH or double MDHs attached to both subunits. In order to examine its organization, the formate oxidation activity and the D-mannitol reduction activity of the FDH-MDH conjugate were individually measured and then fit to respective linear functions relating enzymatic activities to molar concentrations. The FDH-MDH conjugate solution was found to have the formate oxidation activity corresponding to 5.6 μM of a monomeric FDH-AZF, i.e. 2.8 μM of a dimeric FDH-AZF, and the D-fructose reduction activity corresponding to 2.5 μM of MDH-AZF, demonstrating a 2:1 molar species—a dimeric FDH-AZF cross-linked to a single MDH-AZF—was dominant over a 1:1 molar species.

In the presence of a saturating amount of the substrates D-fructose and formate, an efficient transfer of NADH generated by FDH to the active site of MDH is a rate-limiting step in the enzymatic production of D-mannitol (Fig. 1C). In order to investigate the importance of multi-enzyme conjugation on NADH transport, the multi-enzyme cascade reaction was conducted without agitation in the presence of either the FDH-MDH conjugate at a concentration corresponding to 5 nM MDH-AZF or a free enzyme mix of FDH-WT (5.5 nM as a dimer) and MDH-WT (5 nM) as well as an excess of substrates and NAD^+. A low concentration of enzymes and the absence of turbulent stirring should create a locally diffusion-controlled cascade reaction system in which interenzyme transport of NADH determines the rate of mannitol production, thereby facilitating the observation of enhanced catalytic performance contributed by site-specific enzyme tethering. At 3 and 6 hrs after the reaction began, the samples were taken from the reaction solution and subjected to the D-mannitol assay (Fig S3). In the presence of the FDH-MDH conjugate, 25 μM of ... mannitol was produced for 3 hrs, whereas only 13 μM with free enzymes (Fig. 4B). At 6 hr, a similar trend was observed. In the presence of the FDH-MDH conjugate or free enzymes, 42 and 20 μM of D-mannitol were detected, respectively. The apparent ... higher reaction efficiency of the FDH-MDH conjugate over unconjugated enzymes can be attributed to proximity channeling ... NADH between the two enzymes, when there is no stirring to thoroughly mix components in reaction solutions. Regardless of enzyme concentrations, the conjugated FDH has the subsequent enzyme, MDH, in proximity within the spatial radius set by the chemical linker. The proximity effect by the enzyme conjugation, in comparison to free enzymes, became more pronounced when the intermolecular distance was greater (Fig. S4A), or lessened at a higher concentration of enzymes (Fig. S4B). To summarize, these results clearly demonstrated that multiple enzymes were conjugated at specific sites with the retained activities, and the enzyme conjugate showed the enhanced catalytic efficiency over free enzymes by proximity-enhanced NADH processing.

In this study, we developed a strategy to construct the multi-enzyme reaction system using two orthogonal click reactions (SPAAC and IEDDA) as well as site-specific incorporation of a non-natural amino acid (AZF). Introduction of a SPAAC-clickable azide function group to permissive sites of FDH and MDH served as a chemical handle for IEDDA-clickable linker conjugation. Then, the two enzyme-linker conjugates were connected via IEDDA click reaction. The multi-enzyme conjugate (FDH-MDH conjugate) was successfully isolated through standard chromatographic protein purification procedures. The FDH-MDH conjugate exhibited enhanced D-mannitol production rate compared to free FDH and MDH likely due to cofactor shuttling between FDH and MDH. Since

ChemComm Accepted Manuscript

the technique and strategy described here are very general, they would be applicable to conjugation of other multiple enzymes aiming for enhanced catalytic properties. More broadly, this strategy would enable a convenient protein-to-protein conjugation without significant perturbations of protein conformation. Development of new bioorthogonal chemistries[27, 28] will fuel applications of multi-enzyme reaction systems in more diverse fields.

The authors acknowledge financial support from the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (Grant No. 2014R1A2A1A11050322).

Notes and references

1. S. Schoffelen and J. C. van Hest, *Curr Opin Struct Biol*, 2013, **23**, 613-621.
2. R. A. Sheldon, *Appl Microbiol Biotechnol*, 2011, **92**, 467-477.
3. E. Ricca, B. Brucher and J. H. Schrittwieser, *Advanced Synthesis & Catalysis*, 2011, **353**, 2239-2262.
4. A. H. Elcock and J. A. McCammon, *Biochemistry*, 1996, **35**, 12652-12658.
5. H. S. Seo, Y. J. Koo, J. Y. Lim, J. T. Song, C. H. Kim, J. K. Kim, J. S. Lee and Y. D. Choi, *Appl Environ Microbiol*, 2000, **66**, 2484-2490.
6. M. O. Mansson, N. Siegbahn and K. Mosbach, *Proc Natl Acad Sci U S A*, 1983, **80**, 1487-1491.
7. R. A. Sheldon, *Biochem Soc Trans*, 2007, **35**, 1583-1587.
8. D. P. Bloxham, *Biochem J*, 1977, **167**, 201-210.
9. E. Ferrari, M. Soloviev, D. Niranjan, J. Arsenault, C. Gu, Y. Vallis, J. O'Brien and B. Davletov, *Bioconjug Chem*, 2012, **23**, 479-484.
10. E. Steen Redeker, D. T. Ta, D. Cortens, B. Billen, W. Guedens and P. Adriaensens, *Bioconjug Chem*, 2013, **24**, 1761-1777.
11. J. Shimada, T. Maruyama, M. Kitaoka, H. Yoshinaga, K. Nakano, N. Kamiya and M. Goto, *Chemical Communications*, 2012, **48**, 6226-6228.
12. S. Schoffelen, J. Beekwilder, M. F. Debets, D. Bosch and J. C. M. v. Hest, *Bioconjug Chem*, 2013, **24**, 987-996.
13. T. Matsumoto, T. Tanaka and A. Kondo, *Biotechnology Journal*, 2012, **7**, 1137-1146.
14. S. I. Lim, Y. Mizuta, A. Takasu, Y. H. Kim and I. Kwon, *PLoS One*, 2014, **9**, e98403.
15. B. K. Raliski, C. A. Howard and D. D. Young, *Bioconjug Chem*, 2014, **25**, 1916-1920.
16. D. Guan, Y. Kurra, W. Liu and Z. Chen, *Chem Commun (Camb)*, 2015, **51**, 2522-2525.
17. J. C. Wu, C. H. Hutchings, M. J. Lindsay, C. J. Werner and B. C. Bundy, *J Biotechnol*, 2015, **193**, 83-90.
18. S. I. Lim, Y. S. Hahn and I. Kwon, *J Control Release*, 2015, **207**, 93-100.
19. B. C. Bundy and J. R. Swartz, *Bioconjug Chem*, 2010, **21**, 255-263.
20. H. Choe, J. C. Joo, D. H. Cho, M. H. Kim, S. H. Lee, K. D. Jung and Y. H. Kim, *PLoS One*, 2014, **9**, e103111.
21. H. Nanba, Y. Takaoka and J. Hasegawa, *Biosci Biotechnol Biochem*, 2003, **67**, 2145-2153.
22. P. Brunker, J. Altenbuchner, K. D. Kulbe and R. Mattes, *Biochim Biophys Acta*, 1997, **1351**, 157-167.
23. M. Slatner, G. Nagl, D. Haltrich, K. D. Kulbe and B. Nidetzky, *Biocatal Biotransfor*, 1998, **16**, 351-363.
24. S. Ahmad, M. Gromiha, H. Fawareh and A. Sarai, *BMC Bioinformatics*, 2004, **5**, 51.
25. S. I. Lim, Y. Mizuta, A. Takasu, Y. S. Hahn, Y. H. Kim and I. Kwon, *J Control Release*, 2013, **170**, 219-225.
26. S. I. Lim, S. Yoon, Y. H. Kim and I. Kwon, *Molecules (Basel, Switzerland)*, 2015, **20**, 5975-5986.
27. X. Ning, R. P. Temming, J. Dommerholt, J. Guo, D. B. Ania, M. F. Debets, M. A. Wolfert, G.-J. Boons and F. L. van Delft, *Angewandte Chemie International Edition*, 2010, **49**, 3065-3068.
28. A. Borrmann, O. Fatunsin, J. Dommerholt, A. M. Jonker, L. W. P. M. Löwik, J. C. M. van Hest and F. L. van Delft, *Bioconjug Chem*, 2015, **26**, 257-261.

ChemComm Accepted Manuscript

 This journal is © The Royal Society of Chemistry 20xx