Site-specific immobilization of CMP-sialic acid synthetase on magnetic nanoparticles and its use in the synthesis of CMP-sialic acid†

Ching-Ching Yu,‡[a] Po-Chiao Lin‡[ab] and Chun-Cheng Lin*[ab]

Received (in Cambridge, UK) 23rd October 2007, Accepted 7th January 2008
First published as an Advance Article on the web 23rd January 2008
DOI: 10.1039/b716330d

Through the native chemical ligation, CMP-sialic acid synthetase (CSS) was site-specifically immobilized on magnetic nanoparticles and presented excellent enzymatic performance.

The combination of biotechnology and nanotechnology has led to the development of biomolecule-conjugated nanomaterials that are highly specific with regard to intermolecular interactions. These versatile materials have promising applications in drug delivery, cell targeting, protein epitope mapping, and protein–protein interactions.[1] The excellent performance of nanomaterials in bionanotechnology has encouraged scientists to pursue the practical application of these reagents in organic synthesis. Due to their small size and ability to be suspended in reaction media, chemo-catalyst-[2] and biocatalyst-modified[3] nanomaterials result in enhanced reaction efficiency and have the attractive attribute of decreasing environment pollution by reducing the amount of solvents/reagents needed for synthetic reactions.

Enzymes are versatile biocatalysts used in many research areas, including organic synthesis, immunoassay and substrate sensing.[4] The major advantage of using enzymes instead of chemical catalysts is their remarkable chemo-, regio- and stereo-selectivities under mild reaction conditions.[5] However, the reusability and long-term stability of enzymes have limited their applications in organic synthesis. The combination of rapidly developing nanotechnology and the availability of a broad range of enzymes provides a new type of enzymatic platform that has shown superior reusability, stability and efficiency. Recently, various enzymes[3] such as lipase,[6] esterase,[7] protease[8] and lactamase[9] have been covalently linked to nano-scale carriers that present the unique advantages of potential reuse and long-term stability. However, in most of the approaches, enzymes have been attached to the nanomaterial surface through random amide bond or Schiff base formation by using lysine or arginine residues, resulting in significant loss of enzyme activity after immobilization.[6] Although noncovalent immobilization of enzymes with site-specific control, such as the His-tag/Ni interaction, has been used to immobilize enzymes on agarose beads[10] or magnetic nanoparticles (MNPs),[11] the potential dissociation of the enzyme from the solid surface may interfere the long-term storage and reusability of the immobilized enzyme.[12] Thus, development of new enzyme immobilization methods remains of considerable interest in the field of biotechnology for organic synthesis.

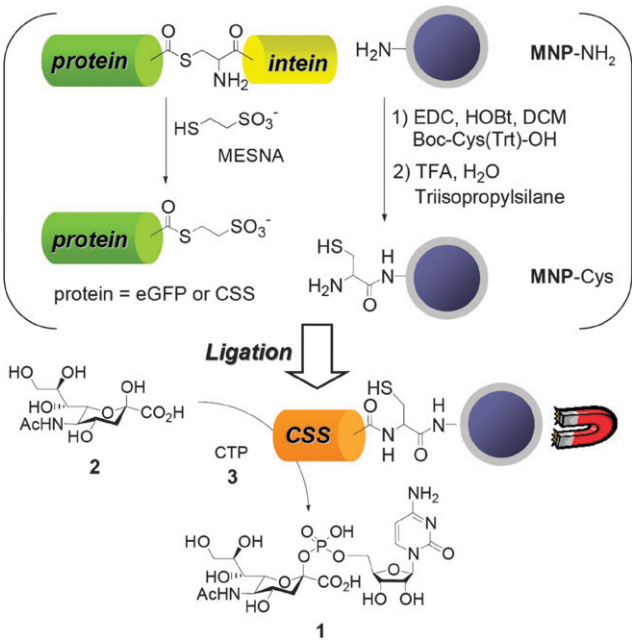
Previously, taking advantage of the large surface area to volume ratio of nanoparticles, we showed that nanoparticles are highly promising as the basis for multivalent ligand carriers[13] that interact rapidly with target substrates in aqueous solution compared with microbeads.[14] We have also demonstrated that the protein binding activity can be maintained on the solid support by site-specific covalent immobilization.[15] Combined with our previous findings, we now describe a simple method to immobilize a target enzyme on MNPs in a site-specific manner using the intein expression system and native chemical ligation (NCL).[16,17] The advantage of this method is that the enzyme is site specifically and covalently immobilized on MNPs without tedious enzyme purification steps; moreover, the resulting complex can be directly separated from solution by a magnet.

As a proof of concept, enhanced green fluorescent protein (eGFP) and CMP-sialic acid synthetase[18] (CSS) were chosen as target proteins and immobilized at their C termini to cysteine-functionalized MNPs (**MNP**-Cys) as illustrated in Scheme 1. **MNP**-Cys was prepared from amine-functionalized iron oxide (**MNP**-NH_2) which was obtained by a conventional sol–gel process.[14b]

MNP-NH_2 was coupled with Boc-Cys(Trt)-OH to give a black powder which was then deprotected by trifluoroacetic acid (TFA) and triisopropylsilane to yield **MNP**-Cys. Meanwhile, target proteins were expressed with the intein fragment at their C termini using the IMPACT system.[15] Initially, unpurified eGFP obtained from expressed cell lysate was used to investigate the efficiency of NCL. We found that reaction at room temperature (25 °C) and in the presence of mercaptoethanesulfonic acid (MESNA) provide better conjugation efficiency than those at 4 °C and without MESNA (see ESI†). To reduce potential non-specific interactions, the eGFP was pre-purified as a MESNA-fused protein by treating MESNA to exchange with intein during affinity separation (Scheme 1). **MNP**-NH_2 or **MNP**-Cys was incubated in the presence of MESNA with either cell lysate of *E. Coli* that expressed eGFP (one-pot procedure) or purified eGFP (two-pot procedure). Then, an equivalent amount of immobilized protein on MNP complex was spotted on a glass slide, and fluorescence emission was detected by a microarray reader. The emission

[a] *Department of Chemistry, National Tsing Hua University, Hsinchu, Taiwan. E-mail: cclin66@mx.nthu.edu.tw; Fax: +886-3-5753147*
[b] *Chemical Biology and Molecular Biophysics, Taiwan International Graduate Program and Genomic Research Center, Academia Sinica, Taipei, Taiwan*

† Electronic supplementary information (ESI) available: Enzymatic reaction conditions and HPLC spectrum. See DOI: 10.1039/b716330d
‡ Authors equal contribution.



Scheme 1 Combination of the intein expression system and native chemical ligation for site-specific and covalent immobilization of target proteins. eGFP: enhanced green fluorescent protein; CSS: CMP-sialic acid synthetase; MNP: magnetic nanoparticle; Cys: cysteine.

intensity showed that when **MNP**-Cys was used, both one- and two-pot procedures yielded comparable emission intensity, indicating that it was not necessary to use purified protein to achieve site-specific immobilization (see ESI†). As expected, no emission was observed when **MNP**-NH_2 was used in either procedure.

The CSS from *Neisseria meningitidis* serogroup B was then used to demonstrate the practical application of the method. CSS catalyzes the formation of CMP-sialic acid **1** from sialic acid **2** by utilizing CTP **3** (Scheme 1). Compared with many hydrolytic enzymes, CSS is less robust and thus its activity may be sensitive to the immobilization method used. The conjugation efficiency was measured as the amount of CSS on MNPs, as determined by the BCA protein assay. The amount of protein per mg enzyme–MNP complex was determined as 94.3 and 67.2 μg for the one- and two-pot procedures, respectively. When the purified CSS was immobilized on **MNP**-NH_2 by random amide bond formation using suberic acid bis-*N*-hydroxysuccinimide ester as the cross linker, 61.9 μg of enzyme was loaded—similar to that measured for the two-pot procedure—indicating that the CSS loading capacity on the particle surface was approximately this amount. SDS-PAGE analysis of the fabricated MNP complex produced by the one-pot procedure revealed smeared bands, indicating that the higher protein loading rate measured for the one-pot procedure may be caused by non-specific absorption of contaminating proteins on the MNP surface (see ESI†). The activity (*i.e.*, per weight of protein) of the immobilized CSS from the one-pot procedure was 63 ± 2% relative to the two-pot procedure (see ESI†), demonstrating that CSS activity was insignificantly affected by the contaminating *E. coli* proteins.

To evaluate the activity of CSS on MNPs, an HPLC assay[19] was used to measure the initial rate of CMP-sialic acid synthesis and the decrement of CTP concentration. CMP-sialic acid synthetase catalyzed reactions were performed in Tris-HCl buffer (pH 8.5) containing $MgCl_2$ and DTT. After addition of CTP and an equimolar amount of sialic acid to the reaction buffer, the pH of solution was readjusted to 8.5 with NaOH. The reaction was initiated by the addition of inorganic pyrophosphatase and CMP-sialic acid synthetase, shaking at 37 °C. The reaction was stopped by addition of SDS. The resultant precipitate was removed by 0.22 μm membrane filter, then analyzed by reverse phased HPLC (see ESI†). In comparison with purified CSS, the relative activity of site-specifically immobilized CSS was 76.8%, whereas that of the randomly immobilized CSS was 33.2%. Otherwise, we also immobilized CSS on magnetic microbead with site-specific manner, and the relative activity was 36.6% (Fig. 1(a)). The X-ray crystal structure of CSS[20] was used to elucidate the enzyme activity loss by random immobilization. The X-ray structure reveals that 5 of 19 lysine and arginine residues on the protein surface are located near the active site. These amino-containing residues may react with activated esters involved in protein immobilization, causing a partial or complete block of the active site (Fig. 1(b)). By contrast, the C terminus of CSS is far from the active site, and thus immobilization at this site would not be expected to impact the structure and, hence, activity of CSS. Our data demonstrate that the site-specific fabrication of **MNP**-CSS preserved enzymatic activity significantly better than the random immobilization method. The observed loss of ~23% of the CSS activity after site-specific immobilization may be caused by the bulky MNP, which may decrease the enzyme dynamics (*i.e.*, a kinetic constraint), resulting in a lower reaction rate. Notably, CSS naturally forms a dimer[20] that exhibits the highest activity, whereas the immobilized enzyme is monomeric and thus would be expected to have somewhat decreased activity. The specific activity of **MNP**-CSS was determined as 62 μmol min^{-1} per mg of CSS, whereas that of purified CSS was 81 μmol min^{-1}, in agreement with the enzyme activity results mentioned above.

Due to the large surface area to volume ratio and stable carrier features of MNPs,[14] we investigated the potential advantages of the **MNP**-CSS for long-term storage, ease of recovery, and reusability. To study the long-term stability of **MNP**-CSS, the same batch of **MNP**-CSS was divided into

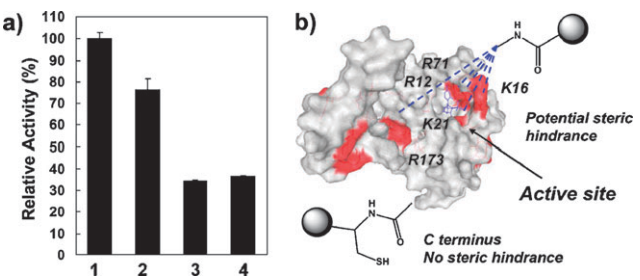


Fig. 1 **MNP**-CSS activity affected by the immobilization method. (a) Relative activity of CSS. Column 1: native CSS, column 2: site-specific immobilized CSS, column 3: random immobilized CSS, column 4: site-specific immobilized CSS on microbeads. (b) Elucidation of steric effect by CSS X-ray structure.

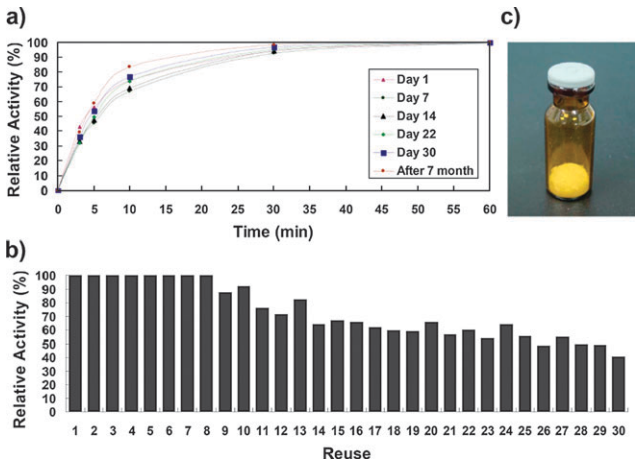


Fig. 2 The stability and reusability of **MNP**-CSS. (a) Stability of **MNP**-CSS. (b) Enzymatic activity of **MNP**-CSS with repetitive use in the enzyme assay. (c) Prepared sample of CMP-sialic acid.

several equivalent aliquots that were stored at 4 °C. At 7-day intervals, an aliquot of MNP-CSS was incubated with freshly prepared reaction solution and the reaction kinetics were measured. As shown in Fig. 2(a), essentially full CSS activity was maintained even after seven months. This long-term stability illustrates the advantage of attaching the enzyme in a chemically specific manner to MNPs. The reusability of **MNP**-CSS was determined by assessing the CSS activity of the same **MNP**-CSS particles in repeated reactions. After being mixed vigorously for 5 min to allow the reaction to proceed, **MNP**-CSS was then separated from solution *via* a magnet, and the remaining solution was analyzed by HPLC to determine the formation of CMP-sialic acid. The **MNP**-CSS was recovered by washing with Tris buffer (pH 8.5) and then used directly for the next identical assay. As shown in Fig. 2(b), repeated use of **MNP**-CSS for ten times had little effect on the observed enzyme activity and maintained about half activity after thirty times operation. To further demonstrate its practical application, **MNP**-CSS successfully used to prepare 367.1 mg CMP-sialic acid with 91% purity at one trial, shown in Fig. 2(c).

In conclusion, using a combination of an intein-fused protein expression technique and NCL, target proteins were covalently immobilized on MNPs in a site-specific manner without tedious protein purification procedures. Due to their nano-scale size, MNPs maximize the surface area available for enzyme attachment, and the MNPs are easily recovered from aqueous suspension by applying a magnetic field. Our **MNP**-CSS preparation retained almost 100% activity even after storage at 4 °C for 7 months or reuse for at least ten consecutive enzyme assays. The site-specific immobilization of CSS on a solid support yielded superior enzyme activity compared with that measured for CSS covalently immobilized using the conventional random linkage technique. Furthermore, we demonstrated that this method could be applied to large-scale reaction with satisfying yield and purity. These features of **MNP**-CSS, and perhaps other MNP-enzyme preparations, make it amenable to applications in organic synthesis.

We thank J.-L. Ko for helpful discussion and Prof. M. Frosch for generously providing us with plasmid pMF32.35. This work was financially supported by the National Tsing Hua University, Academia Sinica and the National Science Council, Taiwan.

Notes and references

1 (*a*) N. L. Rosi and C. A. Mirkin, *Chem. Rev.*, 2005, **105**, 1547; (*b*) E. Katz and I. Willner, *Angew. Chem., Int. Ed.*, 2004, **43**, 6042.
2 A. Hu, G. T. Yee and W. Lin, *J. Am. Chem. Soc.*, 2005, **127**, 12486.
3 J. Kim, J. W. Grate and P. Wang, *Chem. Eng. Sci.*, 2006, **61**, 1017.
4 K. Drauz and H. Waldmann, *Enzyme Catalysis in Organic Synthesis: A Comprehensive Handbook*, Wiley-VCH, Weinheim, 2002, vol. I–III.
5 (*a*) K. M. Koeller and C.-H. Wong, *Nature*, 2001, **409**, 232; (*b*) A. Schmid, J. S. Dordick, B. Hauer, A. Kiener, M. Wubbolt and B. Witholt, *Nature*, 2001, **409**, 258.
6 A. Dyal, K. Loos, M. Noto, S. W. Chang, C. Spagnoli, K. V. P. MShafi, A. Ulman, M. Cowman and R. A. Gross, *J. Am. Chem. Soc.*, 2003, **125**, 1684.
7 H. R. Luckarift, J. C. Spain, R. R. Naik and M. O. Stone, *Nat. Biotechnol.*, 2004, **22**, 211.
8 J. Kim, J. Lee, H. B. Na, B. C. Kim, J. K. Youn, J. H. Kwak, K. Moon, E. Lee, J. Kim, J. Park, A. Dohnalkova, H. G. Park, M. B. Gu, H. N. Chang, J. W. Grate and T. Hyeon, *Small*, 2005, **1**, 1203.
9 X. Gao, K. M. K. Yu, K. Y. Tam and S. C. Tsang, *Chem. Commun.*, 2003, 2998.
10 X. Chen, J. Fang, J. Zhang, Z. Liu, J. Shao, P. Kowal, P. Andreana and P. G. Wang, *J. Am. Chem. Soc.*, 2001, **123**, 2081.
11 A. R. Herdt, B. S. Kim and T. A. Taton, *Bioconjugate Chem.*, 2007, **18**, 183.
12 T. Cha, A. Guo and X.-Y. Zhu, *Proteomics*, 2005, **5**, 416.
13 C.-C. Lin, Y.-C. Yeh, G.-F. Chen, C.-Y. Yang, Y.-C. Chen, Y.-C. Wu and C.-C. Chen, *Chem. Commun.*, 2003, 2920.
14 (*a*) P.-H. Chou, S.-H. Chen, H.-K. Liao, P.-C. Lin, G.-R. Her, A. C.-Y. Lai, J.-H. Chen, C.-C. Lin and Y.-J. Chen, *Anal. Chem.*, 2005, **77**, 5990; (*b*) P.-C. Lin, P.-H. Chou, S.-H. Chen, H.-K. Liao, K.-Y. Wang, Y.-J. Chen and C.-C. Lin, *Small*, 2006, **2**, 485.
15 P.-C. Lin, S.-H. Ueng, M.-C. Tseng, J.-L. Ko, K.-T. Huang, S.-C. Yu, A. K. Adak, Y.-J. Chen and C.-C. Lin, *Angew. Chem., Int. Ed.*, 2006, **45**, 4286.
16 (*a*) C. Ludwig, M. Pfeiff, U. Linne and H. D. Mootz, *Angew. Chem., Int. Ed.*, 2006, **45**, 5218; (*b*) T. W. Muir, *Annu. Rev. Biochem.*, 2003, **72**, 249.
17 J. A. Camarero, Y. Kwon and M. A. Coleman, *J. Am. Chem. Soc.*, 2004, **126**, 14730–14731.
18 M. Knorst and W.-D. Fessner, *Adv. Synth. Catal.*, 2001, **343**, 698.
19 H. Yu, H. Yu, R. Karpel and X. Chen, *Bioorg. Med. Chem.*, 2004, **12**, 6427.
20 S. C. Mosimann, M. Gilbert, D. Dombroswki, R. To, W. Wakarchuk and N. C. J. Strynadka, *J. Biol. Chem.*, 2001, **276**, 8190.

