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Reversible clustering of magnetic nanobiocatalysts for high-performance biocatalysis and easy catalyst recycling†

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Reversible clusters of nanobiocatalysts are developed *via* non-covalent interaction among enzyme-bound iron oxide magnetic nanoparticles. Dissociation of the clusters by shaking during biotransformation enables high catalytic performance, and re-clustering by stopping shaking after reaction allows for easy magnetic separation. The novel concept is demonstrated with alcohol dehydrogenase RDR for the enantioselective reduction of 7-methoxy-2-tetralone.

Immobilization of enzymes on magnetic nanoparticles (MNPs) as nanobiocatalyst has received increasing attention for *in vitro* biotransformation, since nanobiocatalysts could achieve higher enzyme loading due to the higher surface area to volume ratio as well as higher catalytic performance due to higher mass transfer efficiency in reaction medium. Many enzymes were immobilized on the cheap and biocompatible iron oxide MNPs,[1–4] and some of them achieved good activity and stability. However, despite the special magnetic behaviour of MNPs, facile recycling of nanobiocatalysts remains a significant challenge in practical application.

Iron oxide MNPs behave as superparamagnets only when their diameter is below 15 nm, and it is impossible to effectively capture a single MNP of this size even by high gradient magnetic separation (HGMS).[5] One approach to solve this problem is to coat multiple sub-15 nm iron oxide MNPs with a polymer to form a bigger MNP with stable core–shell structure.[4,6] Attaching enzymes on such MNPs with a diameter of 100–200 nm was reported to give good catalytic performance,[4] and the recycling of the nanobiocatalysts was also demonstrated. However, long separation time and strong magnetic field are required for nanobiocatalysts of these sizes, and it is difficult to achieve complete separation. On the other hand, clustering of sub-15 nm iron oxide MNPs by other methods as nanoclusters is known,[7] and a diameter of >500 nm was thought to be suitable for separation under low to moderate magnetic field strength.[8] Fabrication of biocatalysts with such nanoclusters requires further coating, functionalization, and immobilization with enzymes, giving the final catalyst with too large size and no advantages of nanobiocatalysts. It is a dilemma in the development of nanobiocatalysts: while high enzyme loading and high catalytic performance require small size of catalyst, easy and complete separation needs big size of catalyst.

Herein, we report a novel concept and practical method to solve this challenging problem *via* reversible clustering of magnetic nanobiocatalysts. In this concept, nanobiocatalysts with high enzyme loading are designed to form reversible clusters of micro-size *via* non-covalent interaction among enzyme-bound MNPs (Fig. 1); the clusters are easily dissociated into individual nanobiocatalysts in reaction medium by gentle shaking to catalyze the desired transformation with high performance; and the nanobiocatalysts quickly form clusters again after reaction by stopping shaking, allowing for easy, fast, and complete separation of the catalysts.

The route for preparing the reversibly clustered magnetic nanobiocatalysts is outlined in Fig. 1. GMA-MNPs with a diameter of 62 nm (TEM) containing multiple iron oxide MNPs (7 nm, TEM) as core, poly(glycidyl methacrylate)

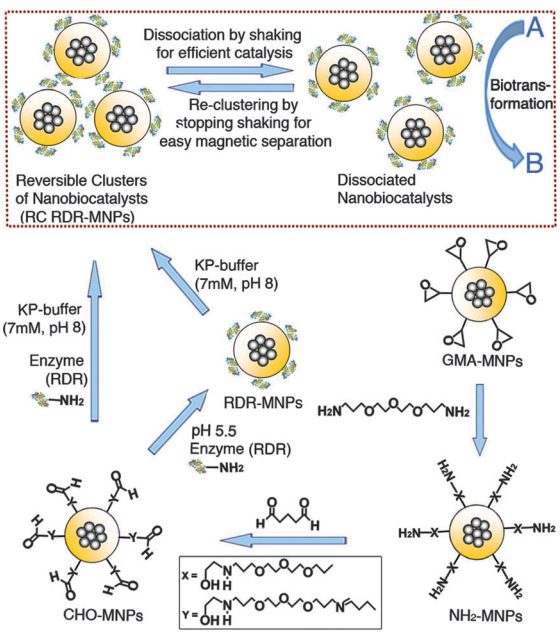

Fig. 1 Synthesis and biocatalysis of reversibly clustered magnetic nanobiocatalysts.

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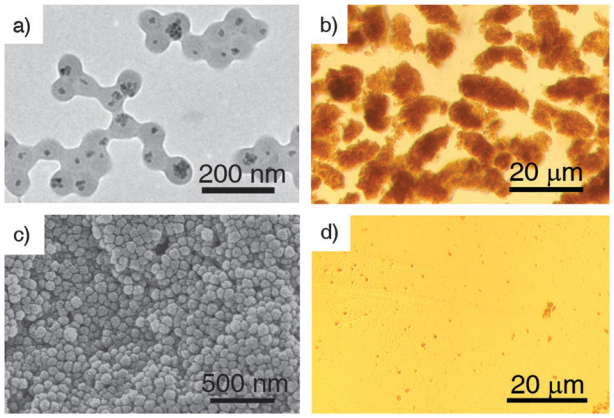

Fig. 2 (a) TEM of CHO-MNPs. (b) Optical microscopy of RC RDR-MNPs. (c) FESEM of RC RDR-MNPs. (d) Optical microscopy of RC RDR-MNPs after shaking at 300 rpm and 30 °C for 2 min.

(PGMA) as shell, and epoxy group as surface function were prepared as the starting material.[4] 4,7,10-Trioxa-1,13-tridecanediamine was reacted with GMA-MNPs to give NH_2-MNPs containing amine groups on the surface, followed by the reaction with glutaraldehyde to afford CHO-MNPs containing aldehyde surface groups. CHO-MNPs were found to have a diameter of 63 nm by TEM (Fig. 2a) and a hydrodynamic size of 157 nm by DLS (pH 7.0). Importantly, CHO-MNPs have zero charge at a pH of 8 (Fig. S5, ESI†), providing the possibility of forming clusters after enzyme immobilization. The size of CHO-MNPs at this pH is 255 nm (DLS), small enough to achieve high enzyme loading and high mass transfer efficiency. The long bridge between the aldehyde group and the surface of CHO-MNPs could contribute to retaining the conformation and activity of the free enzyme. Alcohol dehydrogenase RDR (Molecular weight of 28 KD) from *Devosia riboflavina*[9] was chosen as a model enzyme for demonstrating the concept, since it is a useful enzyme for the enantioselective reduction of ketones to prepare enantiopure alcohols. To immobilize the enzyme, His-tagged RDR[10] was shaken with CHO-MNPs in potassium phosphate (KP) buffer (7 mM) at a pH of 8 and 4 °C for 4 h, which resulted in reversibly clustered RDR-MNPs (RC RDR-MNPs). The overall synthesis of the nanobiocatalyst clusters is simple and highly reproducible, with 89% yield from GMA-MNPs, 76% enzyme loading efficiency, and a specific enzyme loading of 53 mg RDR per g particles. No enzyme was leached during washing or sonication, suggesting that the enzyme was covalently bound to the MNPs.

RC RDR-MNPs have a mean size of 8.5 μm (Fig. 2b) and contain individual nanobiocatalysts with a diameter of 68 nm in the clusters (Fig. 2c). Sonication of RC RDR-MNPs in KP buffer for 15 min gave monodispersed individual nanobiocatalysts with a diameter of 70 nm (Fig. S9, ESI†), suggesting that the individual nanobiocatalysts in the clusters were not cross-linked. Dissociation of the clusters was also easily achieved by shaking the suspension of RC RDR-MNPs in KP buffer at 300 rpm and 30 °C for 2 min. As shown in the microscopy image (Fig. 2d), all big particles disappeared and only several particles with a size of 200–700 nm were left (particles with a size less than 200 nm are invisible in the microscopy image due to the detection limit). Accordingly, a gentle shaking force routinely used in practical biotransformation was enough to dissociate the clusters. After stopping shaking, the nanoparticles clustered again within 3 min to give a mean size of 4.8 μm.

Immobilization of His-tagged RDR with CHO-MNPs at a pH of 5.5 in KP buffer (7 mM) did not give RC RDR-MNPs, but formed fine nanobiocatalysts (RDR-MNPs) with a diameter of 65 nm (FESEM). Adjusting the pH to 8 resulted in the fast clustering of RDR-MNPs within 5 min. This clearly demonstrated that a pH of 8 is essential for the clustering. At this pH, CHO-MNPs have zero charge, which gives insufficient electrostatic repulsion to counteract the attraction and aggregation caused by van der Waals forces. The presence of enzymes on MNPs is also necessary for the clustering: shaking of CHO-MNPs in KP buffer (0 to 90 mM) at a pH of 8 in the absence of enzymes did not result in any clusters. The enzymes could contribute to the clustering by the attractive forces between enzymes through permanent and induced dipoles. Moreover, salts help the clustering by decreasing the hydration layer around the enzymes and increasing hydrophobic interactions between enzymes. At a pH of 8.0, immobilization of His-tagged RDR with CHO-MNPs in KP buffer at a KP concentration lower than 2 mM did not give RC RDR-MNPs. However, increase of KP concentration to 7 mM resulted in fast formation of the clusters of nanobiocatalysts.

The easy separation of RC RDR-MNPs under a magnet is demonstrated in Fig. 3a and b: complete separation was achieved within only 4 s. In comparison, the separation of fine RDR-MNPs under a magnet was not complete even after 20 min (Fig. 3c and d), as evidenced by the brown yellow colour of the solution that contains some nanoparticles. RC RDR-MNPs are superparamagnetic with a saturated magnetization of 11 emu per g particles, similar to the fine RDR-MNPs, shown in the vibrating sample magnetometry (VSM) of Fig. 3e and f. Accordingly, the spins on neighboring particles inside the clusters were not close enough to influence the magnetization per mass of particles.[11]

The catalytic performance of RC RDR-MNPs was examined with enantioselective reduction of 7-methoxy 2-tetralone to produce (*R*)-7-methoxy-2-tetralol (Fig. 4a), a useful and valuable pharmaceutical intermediate.[12] As the enzymatic reduction is dependent on the expensive cofactor NADH, isopropanol was used as "the couple substrate" to regenerate the cofactor.

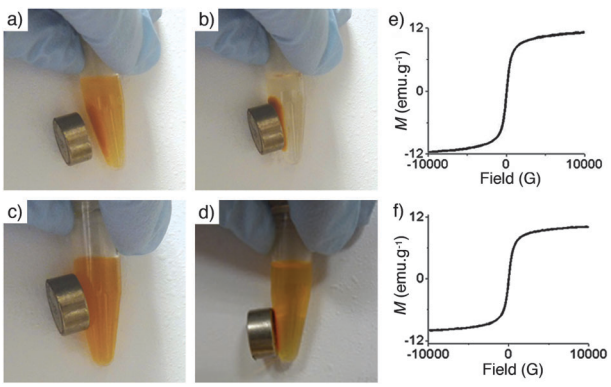

Fig. 3 (a and b) Magnetic separation of RC RDR-MNPs: (a) $t = 0$, (b) $t = 4$ s. (c and d) Magnetic separation of fine RDR-MNPs: (c) $t = 0$, (d) $t = 20$ min. (e) VSM of RC RDR-MNPs. (f) VSM of fine RDR-MNPs.

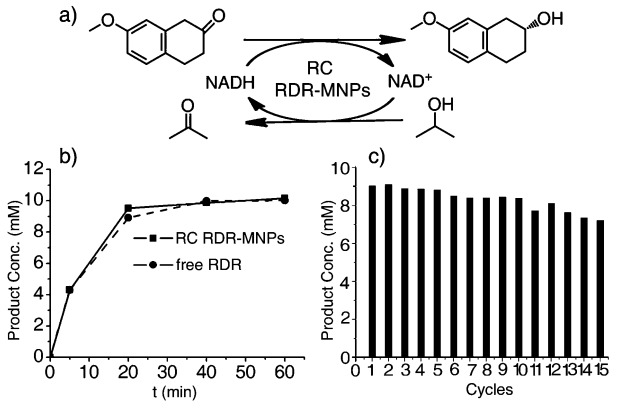

Fig. 4 (a) Scheme of enantioselective bioreduction of 7-methoxy-2-tetralone with cofactor recycling. (b) Time course of the biotransformation at a pH of 8 with RC RDR-MNPs and free RDR, respectively. (c) Recycling of RC RDR-MNPs in the enantioselective bioreduction.

RC RDR-MNPs or free His-tagged RDR was used at 0.1 mg protein per mL for the reduction of 10.5 mM 7-methoxy-2-tetralone, with the addition of 0.0012 mM NADH and 48 mM isopropanol, in Tris buffer (6 mM, pH 8) at 300 rpm and 30 °C for 60 min. As shown in Fig. 4b, RC RDR-MNPs retained 100% activity of the free enzyme, which is better than any reported nanobiocatalysts coated with a dehydrogenase.[3] After 60 min, 7-methoxy-2-tetralol was produced in 97% yield (10.2 mM) with RC RDR-MNPs as the biocatalyst. The enantioselectivity of RC RDR-MNPs was also the same as that of the free enzyme, giving the product in >99% ee (*R*). Furthermore, NADH was efficiently regenerated for 8500 times, which is practical for the synthesis of the chiral pharmaceutical intermediate. These results demonstrated the high performance of the nanobiocatalyst clusters in biotransformation. They also indicated no significant change in the active site of the enzyme before and after immobilization and clustering.

Recycling of RC RDR-MNPs was conducted in the same biotransformation. After 20 min reaction, RC RDR-MNPs were quickly separated under a magnet, washed, and then reused for a new cycle of reaction. As shown in Fig. 4c, the catalyst retained 80% of its original activity after recycling 14 times. This result is much better than those from any reported dehydrogenases immobilized on solid supports.[13] In total, 125 mM of (*R*)-7-methoxy-2-tetralol was produced in >99% ee and 80% yield, by recycling NADH 6000–7700 times in each cycle.

RC RDR-MNPs showed much higher tolerance against lower pH than the free enzyme and retained nearly the same productivity even at pH 5.5 (Fig. S15a, ESI†). They were also much more thermostable than the free enzyme. After 12 h pre-incubation at 70 °C, the free enzyme dropped by 95% in productivity, while RC RDR-MNPs lost only 6% of their original productivity (Fig. S15b, ESI†). These improved stabilities are probably due to the covalent attachment of enzymes on the nanocarriers.

The generality of the concept was demonstrated by using *Thermomyces lanuginosus* lipase (TLL). The reversible cluster RC TLL-MNPs were fabricated in 89% yield, with 42 mg enzyme per g particles and a mean size of 27 μm. They were easily dissociated by shaking, showing 93% activity of the free enzyme for hydrolysing *p*-nitrophenyl butyrate. Clusters were quickly formed after stopping shaking and completely separated within 4 s under the magnetic field. RC TLL-MNPs were also successfully recycled 10 times while retaining 92% activity (see ESI†).

A novel concept of reversible clustering of magnetic nanobiocatalysts for high-performance biocatalysis and easy catalyst recycling is successfully developed. Nanobiocatalysts form reversible clusters *via* interactions between enzymes immobilized on neutrally charged iron oxide MNPs. The clusters are easily dissociated into individual nanobiocatalysts by gentle shaking for efficient biotransformation, and the nanobiocatalysts re-cluster quickly by stopping shaking for easy, fast, and complete separation under a magnet. The concept is proven by using an alcohol dehydrogenase (RDR). The nanobiocatalyst clusters RC-RDR-MNPs are prepared in high yields with high enzyme loading and demonstrate 100% activity and the same enantioselectivity of the free enzyme in bioreduction. They are effectively recycled 14 times to produce (*R*)-7-methoxy-2-tetralol in >99% ee and 125 mM while retaining 80% of the original productivity. The concept has been proven general with *T. lanuginosus* lipase (TLL) and could open an avenue for developing practical *in vitro* biotransformations for different types of enzymatic reactions for green chemical syntheses.

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