ChemComm

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Sputtering deposition of magnetic Ni nanoparticles directly onto an enzyme surface: a novel method to obtain a magnetic biocatalyst†

Cite this: *Chem. Commun.*, 2013, **49**, 1273

Received 11th November 2012,
Accepted 23rd December 2012

DOI: 10.1039/c2cc38737a

www.rsc.org/chemcomm

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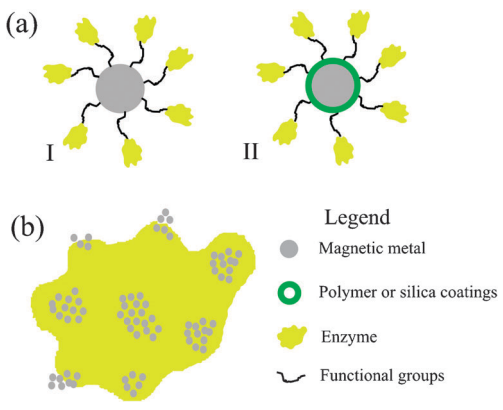
A simple one-step method based on the sputtering deposition of Ni nanoparticles (NP) has been developed for the production of magnetic biocatalysts, avoiding the complications and drawbacks of methods based on chemical functionalisation or coating of magnetic NP. This new technique provided high levels of recovery, reusability and catalytic activity for the lipase–Ni biocatalyst.

Enzymes are versatile biocatalysts that effectively control specific chemical reactions *in vivo* and *in vitro*.[1] They present high catalytic efficiency and selectivity, operate under mild conditions and produce fewer by-products than standard chemical catalysts.[2,3] Compared with current chemical methodologies, enzymes are environmentally friendlier, more energy-efficient and potentially more cost-effective catalysts due to low energy demand operation and easier downstream processing.[4] However, biocatalysts composed only of free enzymes are expensive, present poor long-term stability under processing conditions and are difficult to recover and recycle from the reaction mixture.[2,3,5] In order to overcome these deficiencies, various strategies have been employed to improve biocatalyst functionality, stability and reusability by immobilizing enzymes onto carriers through chemical methods.[3] Immobilization of enzymes onto insoluble organic or inorganic supports maintains the natural catalytic activity and allows biocatalyst separation from reaction systems for recycling, to reduce operational costs and to mitigate enzyme contamination.[2,4] A large number of research reports have shown that immobilized enzymes may have better performance than free enzymes in many aspects.[1–9]

In recent years, the use of magnetic NP for enzyme immobilization has received considerable attention not only due to the possibility of achieving very high surface areas, but also because they provide an easy way to separate enzymes from a reaction mixture by the application of an external magnetic field. These features diminish costs and enhance the product's purity.[1,5–7,10] Most of the techniques of enzyme immobilization onto magnetic NP involve modifications of the NP surface in order to chemically bind or strongly adsorb the enzyme (Scheme 1aI).[3,6] Magnetic carriers are generally coated with polymeric materials, and these over-layers are also modified with various functional groups such as hydroxyl, amino or sulfhydryl groups by copolymerization or chemical surface modification (Scheme 1aII).[4,7,9]

These functional over-layers on magnetic supports can be detrimental to catalytic activity, since multipoint covalent attachments may promote rigidification of the molecular structure of the immobilized enzyme.[5,8] Activity loss due to the restriction of conformational changes after immobilization remains a great challenge to be solved in enzyme engineering, making the development of new immobilization methods one of the main subjects of research in this field.[6,7]



Scheme 1 Techniques of enzyme immobilization onto magnetic NP: enzymes chemically bound or strongly adsorbed on the functionalized surface of the NP (aI) and enzymes chemically bound on the functionalized surface of the NP coated with polymeric materials (aII). Decoration of magnetic particles in enzymes by sputtering (b).

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† Electronic supplementary information (ESI) available: Experimental details. See DOI: 10.1039/c2cc38737a

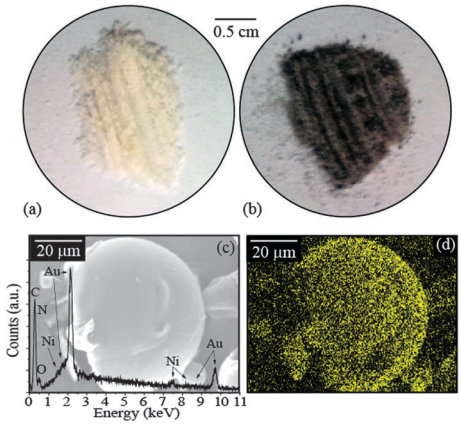

Fig. 1 Optical images showing the lipase powder before (a) and after (b) sputtering deposition of Ni. SEM image and EDS of the product after deposition of Ni (c). Mapping for Ni (d).

In this work, a new approach was developed that enables enzymes to be decorated with magnetic NP[11–14] through a simple physical procedure allowing biocatalyst recovery and reuse in the process. The technique used was the sputtering deposition[15] of magnetic particles directly onto the enzyme surface, without the need for any chemical coating or modification of the NP surface. The performance of a nanocomposite biocatalyst (lipase from *Pseudomonas cepacia* decorated with Ni particles) produced by this method was investigated. Excellent retention of the particles on the enzyme and good levels of functional activity were obtained, even after several cycles of use, as discussed below. Details of magnetic biocatalyst preparation and characterization can be found in detail in ESI.†

Fig. 1a and b show optical images of the lipase powder before and after sputtering deposition of Ni. As can be seen, the whitish color of the lipase became dark after the deposition of Ni. In order to confirm the presence of nickel on the lipase, scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) analyses were carried out on the processed samples (Fig. 1c and d). The Kα characteristic emission line of Ni at about 7.4 keV could be seen in the EDS. Ni was uniformly distributed on the lipase surface as evidenced by the chemical mapping (Fig. 1d).

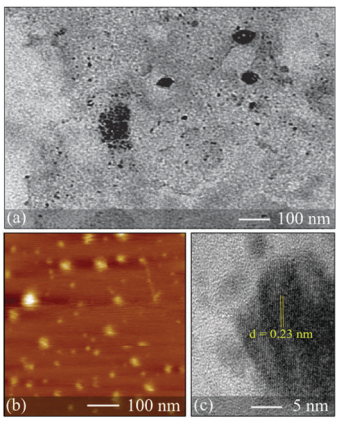

Fig. 2 TEM (a) and AFM (b) images of the lipase after Ni deposition. HRTEM of one of these Ni NP (c).

Fig. 2 shows a more detailed view of the arrangement of the Ni NP on the lipase surface. Both TEM (Fig. 2a) and AFM (Fig. 2b) images indicate the presence of aggregates of Ni particles (black circular regions in the TEM image and white spots in the AFM panels) about 50 nm in diameter and a distribution of smaller particles (~10–20 nm) on top of the amorphous enzyme matrix. High resolution (HR) TEM of one of these particles (Fig. 2c) revealed an inter-planar distance of 0.23 nm, corresponding to the (010) plane of the Ni lattice, which was also confirmed by the Fourier transform of the HRTEM image. The micrographs in Fig. 2a and b also demonstrate the uniform dispersion of the Ni particles, achieved even at the sub-micrometer scale. The quantity of Ni deposited onto the lipase, determined by flame atomic absorption spectrometry, was 1.1 mg of nickel for 1 g of the magnetic biocatalyst. As shown below, this amount was sufficiently low to allow the substrate access to the enzyme's active site, but high enough for the recovery of the enzyme using a magnetic field.

The magnetization curve of the catalyst measured at room temperature using a superconducting quantum interference device (SQUID) magnetometer is shown in Fig. 3a. The free lipase showed a diamagnetic response, as expected. The lipase–Ni composite magnetization curve showed typical ferromagnetic hysteresis with a well-defined "loop". Since the lipase mass does not contribute to magnetization, the Ni content obtained by atomic absorption was used to estimate the saturation magnetization of the biocatalyst; this was found to be around 4.0 emu g^{-1}. For bulk Ni at room temperature, the saturation magnetization is 54.4 emu g^{-1}.[16] Such a notable reduction in magnetization compared to the bulk value is often observed in NP due to the large percentage of surface spins that have a disordered magnetization orientation.[17] In addition, the data showed a remanence of 1.9 emu g^{-1} (about 47% of saturation) and a coercive field of about 58 Oe. Since both values are small but non-zero, these results imply that the mean size of the NP is above the critical diameter for superparamagnetism (around 15 nm for Ni),[16] in accordance with the TEM and AFM findings. Note that sample magnetization saturated with a relatively small external magnetic field (less than 1000 Oe), meaning that strong magnets are not required to recycle the biocatalyst, Fig. 3b.

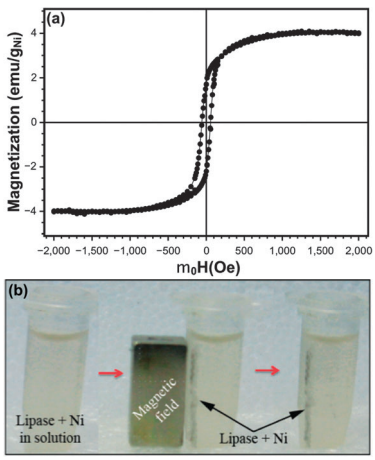

Fig. 3 Magnetization curve of the biocatalyst as a function of the external magnetic field (a). Magnetic biocatalyst dispersed in aqueous solution being recycled for reuse by applying a magnetic field (b).

Table 1 Hydrolysis activity of the biocatalyst

Batch reactions	Biocatalyst	Hydrolytic activity (U g^{-1} of lipase)	Relative activity (%)	Ni content[a] (μg of Ni)
—	Free lipase	339	—	—
1	Lipase + Ni	232	100	2.2
2	Lipase + Ni	230	99.6 ± 0	<LOQ
3	Lipase + Ni	224	96.6 ± 1.4	<LOQ
4	Lipase + Ni	220	94.6 ± 1.7	<LOQ
5	Lipase + Ni	203	87.5 ± 0.4	<LOQ
6	Lipase + Ni	185	79.7 ± 0.7	<LOQ
7	Lipase + Ni	150	64.7 ± 3.2	<LOQ
8	Lipase + Ni	139	60.0 ± 2.7	<LOQ
9	Lipase + Ni	102	44.1 ± 0.9	<LOQ
10	Lipase + Ni	63	27.4 ± 3.4	<LOQ
11	Ni	0	—	—

[a] Limit of quantification (LOQ) 0.12 μg of Ni.

Table 2 Transesterification activity of the biocatalyst

Batch reactions	Biocatalyst	Total conversion (%)	Relative activity (%)	Ni content[a] (μg of Ni)
—	Free lipase	99	—	—
1	Lipase + Ni	78	100	<LOQ
2	Lipase + Ni	76	97.4	<LOQ
3	Lipase + Ni	68	87.2	<LOQ
4	Lipase + Ni	67	85.9	<LOQ
5	Ni	0	—	—

[a] Limit of quantification (LOQ) 0.12 μg of Ni.

Experiments were performed to investigate the hydrolytic and transesterification catalytic activity of the pristine and recycled magnetic biocatalyst (Tables 1 and 2). The magnetic biocatalyst retained 85% of its hydrolytic activity (232 U g^{-1} of lipase) and 79% of its transesterification activity (78% total conversion) compared with the free enzyme. For the hydrolytic reaction, over 60% of the residual activity was retained after eight repeated batch reactions (Table 1). Concerning the transesterification reaction, over 85% of the activity was retained after five repeated batch reactions (Table 2). Moreover, only in the first run a small amount of Ni (2.19 μg) was released into the aqueous medium after hydrolysis and in the organic phase after transesterification with the reuse of the magnetic biocatalyst (Tables 1 and 2).

Our results were also compared to data from the literature in which enzymes were *chemically* immobilized onto magnetic particles. Lipase from *Candida rugosa* immobilized onto Fe_3O_4 magnetic NP supported in ionic liquids *via* adsorption presented 64% immobilization efficiency.[6]

Functionalized Fe_3O_4 magnetic NP were used to immobilize lipase by electrostatic adsorption and covalent binding,[3] retaining 70% of its hydrolytic activity. These results suggest that covalent bond formation *via* epoxy groups and amino groups reduces the conformational flexibility. A 37% decrease in immobilized enzyme activity was also observed for an esterase immobilized on Fe_3O_4 magnetic NP *via* a glutaraldehyde coupling reaction.[9] Covalent attachment promotes rigidification of the molecular structure of the immobilized enzyme.[5,8] In our work, the magnetic biocatalyst retained 85% of its hydrolytic activity and the remaining activity was about 80% after five reuses. The amount of total protein released into the aqueous medium after hydrolysis was observed only after the first two recycles (4.1%). The thermal stability of our catalytic system (see ESI†) was studied and its activity was found to be maintained up to 70 °C of incubation temperature. Moreover, we have found that the catalytic system maintains 78% of its activity after 10 months of storage at 4 °C whereas the free lipase maintains only 52% of its original activity. Therefore it is probable that the Ni itself is not the main responsible for the de-activation processes and the de-activation is related to other phenomena as suggested for related systems such as inhibition of the active site by the reaction media or even enzyme unfolding.[5,8] Finally it is important to note that the magnetization properties of the catalytic system are not affected by its re-use.

We have shown that a simple one-step physical deposition method can be applied for the production of magnetic biocatalysts, avoiding the complications and drawbacks of methods based on chemical functionalisation or coating of magnetic NP. This new technique provided high levels of recovery, reusability and catalytic activity for the lipase–Ni system in both hydrolytic and synthetic reactions. Such behavior suggests that the sputter deposition method prevents, to a large extent, structure stiffening after immobilization, allowing the conformational changes necessary for the transition from the inactive to the active form of the enzyme. This new approach can be further improved and easily expanded to other kinds of magnetic NP and enzymes. It may provide a simple and inexpensive strategy for the production of efficient and recyclable magnetic biocatalysts with potential use on the industrial scale.

Thanks are due to CNPq, MCT-INCT, CAPES and FAPERGS.

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