ChemComm

COMMUNICATION

Enzyme immobilised novel core–shell superparamagnetic nanocomposites for enantioselective formation of 4-(R)-hydroxycyclopent-2-en-1-(S)-acetate†

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Lipase immobilized novel high surface area core–shell superpara-magnetic nanoparticles have been fabricated and used as efficient reusable catalysts for the selective production of pharmaceutically important chiral isomers from *meso*-cyclopent-2-en-1,4-diacetate.

Enzymes are well known as catalysts for various bio-transformations however, it is difficult to separate the expensive enzymes from the reaction mixtures. Hence immobilization of enzymes on solid supports such as zeolites,[1] agarose beads,[2] epoxy resins[3] and porous glass[4] is an important step for the recovery and recycling of the enzymes. Immobilization of lipase in magnetisable solid supports has recently been exploited[5,6] for the ester hydrolysis due to an additional advantage of easy recovery of expensive enzymes from the reaction mixture by a single step separation under the influence of an external magnetic field. The use of immobilised enzymes is particularly important as they exhibit increased stability and catalytic activity compared to free enzymes.[7] Immobilization of an enzyme is important as that leads to the enzyme being dispersed over a large surface area, enhancing mass transfer and preventing aggregation.[8] Catalytic hydrolysis of *meso*-cyclopent-2-en-1,4 diacetate has been reported previously using *Mucor* sp.,[9] pancreatin[10] and *Mucor miehei* lipase (Chirazyme),[11] however recovery of enzymes from the reaction mixture was a great concern and drawback to the technology. Herein, we report (a) fabrication of a high surface area novel core–shell superparamagnetic nanoparticles (b) surface modification of core–shell nanoparticles with aminopropyl tri-ethoxy silane by recently
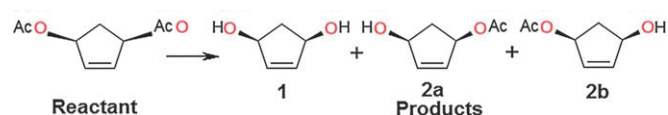
Scheme 1 Enzymatic hydrolysis of *meso*-cyclopent-2-en-1,4-diacetate to chiral products (**2a** and **2b**).

developed Tri-phasic Reverse Emulsion (TPRE)[12] method for the immobilisation of *Pseudomonas Fluorescens Lipase* (PFL) and *Candida Rugosa Lipase* (CRL) by covalent coupling (c) one step catalytic conversion (see reaction Scheme 1) of *meso*-cyclopent-2-en-1,4 diacetate to important optical isomers such as 4-(R)-hydroxycyclopent-2-en-1-(S)-acetate and 4-(S)-hydroxycyclopent-2-en-1-(R)-acetate by the careful selection of a suitable lipase enzyme with a single step separation of the expensive catalyst by magnetic separation.

Superparamagnetic iron oxide nanoparticles (SPIONs) with narrow particle size distribution with an average diameter of 10 nm were synthesized by a co-precipitation method (see ESI†) of ferrous and ferric chloride salts following our previously reported protocol.[13] SPIONs were then coated with a thick mesoporous silica shell using tetraethyl orthosilicate (TEOS) as a silica source, cetyl-trimethyl ammonium bromide (CTAB) as a structure directing template in an alkaline medium (NH$_4$OH) by a modified protocol (see details in ESI†) from our earlier report.[14]

Fig. 1a presents the TEM image of the core–shell nano-particles of around 270 nm in diameter where the core was formed with multiple SPIONs of each approximately 10 nm diameter and mesoporous silica shell (thickness ∼ 135 nm) with mesopore diameter of approximately 3.5 nm. Small Angle X-ray Scattering (SAXS) data (see Fig. 1b and ESI,† Fig. S1) indicate the presence of mesopores of repeating distance of 3.7 nm which is similar to the mesopore diameter measured using TEM (Fig. 1a and see ESI,† Fig. S2) and nitrogen gas adsorption (Fig. 1c). The XRD pattern (see Fig. S3 in ESI†) of core iron oxide nanoparticles indicated the magnetite (Fe$_3$O$_4$) phase due to the characteristic finger print patterns. N$_2$ gas adsorption analysis (Fig. 1c) shows a typical type IV isotherm with a hysteresis loop confirming the mesoporous structure.

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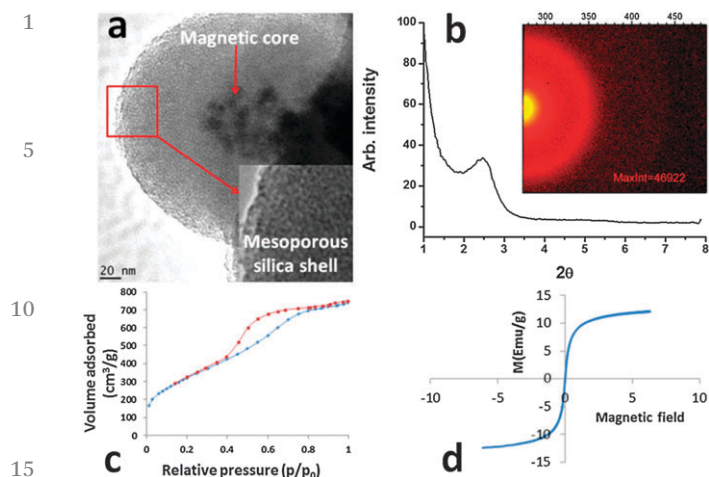
Fig. 1 TEM image (a), small angle X-ray scattering (b), N$_2$ adsorption–desorption isotherm (c) and magnetic measurement (d) of novel core–shell mesoporous superparamagnetic nanoparticles.

Fig. 2 Conversion of reactant and the formation of products in % at different reaction times by enzymatic hydrolysis by using free PFL (a), physically adsorbed PFL on core–shell nanoparticles (b), PFL immobilised core–shell nanoparticles by covalent coupling via TPRE approach (c), and % conversion of reactant during the reusability test (d).

The BJH adsorption average pore diameter (see ESI,† pages S9–S13) of approximately 4 nm and the BET surface area was calculated to be 1187.8 m^2 g^{-1}. The exceptional high surface area compared to our previously reported core–shell mesoporous silica–magnetite[14,15] is the key in our study as a novel catalytic support. Fig. 1d presents the magnetic data using variable sample magnetometer (VSM). Zero remanence ($M_R = 0$) is an indication of superparamagnetism with low saturation magnetization value of approximately 12 emu g^{-1}. The low saturation magnetization value compared to a large value ($\sim$80 emu g^{-1}) of pure superparamagnetic magnetite nanoparticles can be due to the presence of a thick diamagnetic silica shell similar to our earlier report.[15]

Surface functionalisation of the core–shell nanoparticles with hydrophilic silica surface was carried out using aminopropyl triethoxy silane using novel TPRE method[12] (see ESI† for details) in order to create uniform layer of terminal –NH$_2$ functional groups. The –NH$_2$ groups were further converted to terminal aldehyde (–CHO) groups by reacting with glutaraldehyde solution in SSC buffer as reported earlier.[6,12] The surface –CHO modified nanoparticles were covalently coupled (see ESI† for experimental protocol) with –NH$_2$ groups of lipase (PFL and CRL) enzymes. The reaction scheme for the immobilisation of *lipase* with surface –CHO groups has been reported[6] earlier by our group using a different materials *i.e.* hierarchically ordered porous solid (HOPS).

The lipase immobilised core–shell nanoparticles and free lipases were used for the enzymatic hydrolysis of *meso*-cyclopent-2-en-1,4-diacetate under a biphasic reaction condition using a solvent mixture (80% hexane and 20% water) at 25 °C with end-over-end rotation (40 rpm) for up to 48 hours reaction time (see ESI† for experimental details). A biphasic solvent was used in our experiment as the products are soluble in water and insoluble in hexane whereas the reactant is mostly soluble in hexane. Recently our group has established[16] that a bi-phasic system is powerful compared to a single phase system for trans-esterification reaction using free lipase as catalyst. The activity of free and immobilised lipases in the conversion of *meso*-cyclopent-2-en-1,4-diacetate (reactant) and the
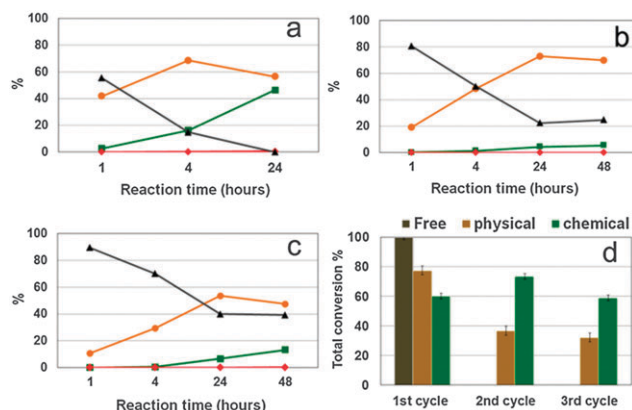
formation of products (see reaction Scheme 1) were determined by GC and GCMS analysis using a pre-established calibration curves (see ESI,† Fig. S4 to S7).

Fig. 2 and 3(a to c) present the percentage (%) of unreacted *meso*-cyclopent-2-en-1,4-diacetate (black lines) and the corresponding products such as 4-(R)-hydroxycyclopent-2-en-1-(S)-acetate (orange), 4-(S)-hydroxycyclopent-2-en-1-(R)-acetate (red) and cyclopent-2-en-1,4-diol (green) at different time intervals (1, 4, 24 and 48 h) by enzymatic hydrolysis using PFL (Fig. 2) and CRL (Fig. 3). Fig. 2d and 3d exhibit the percentage conversion of reactant during the reusability test (cycles 1 to 3). Free enzyme couldn't be recovered from the reaction mixture hence presented as one cycle (grey bar in Fig. 2d and 3d).

Percentage (%) conversion of reactant to various products increases with increasing the reaction times from 1 to 24 h for all materials using free enzymes or enzymes immobilised nanoparticles. Free PFL performed a complete conversion (100 μmol of reactant per mg of enzyme) of reactant to products
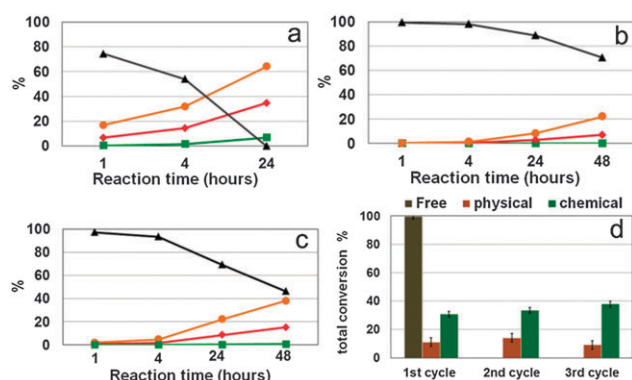

Fig. 3 Conversion of reactant and the formation of products in % at different reaction times by enzymatic hydrolysis using free CRL (a), physically adsorbed CRL on core–shell nanoparticles (b), CRL immobilised on core–shell nanoparticles by covalent coupling via TPRE approach, and % conversion of reactant during the reusability test using three different materials (d).

after 24 h of reaction whereas PFL immobilized (physically or chemically) nanoparticles performed a partial conversion. Physically adsorbed PFL materials performed around 78% conversion of reactant (78 μmol of reactant per mg of enzyme) whereas chemically immobilized PFL materials performed around 60% conversion of reactant (60 μmol of reactant per mg of enzyme). It is important to mention that free PFL or PFL immobilized (physically or chemically) nanoparticles produced only 4-(R)-hydroxycyclopent-2-en-1-(S)-acetate (**2a** of Scheme 1) without the formation of 4-(S)-hydroxycyclopent-2-en-1-(R)-acetate (**2b** of Scheme 1). Increasing the reaction times from 1 to 24 h, the concentration of 4-(R)-hydroxycyclopent-2-en-1-(S)-acetate decreases and the concentration of unwanted cyclopent-2-en-1,4-diol (see Scheme 1, product **1**) increases due to the further hydrolysis of acetate group to alcohol group. Physically adsorbed PFL nanoparticles performed better in catalytic conversion of reactant to desired product 4-(R)-hydroxycyclopent-2-en-1-(S)-acetate, however when re-used the catalyst, the catalytic conversion reduced more than 50% in the 2nd and 3rd cycles whereas chemically immobilized PFL exhibited a stable catalytic activity. The higher catalytic activity of physically adsorbed PFL in the 1st cycle could be due to the presence of free enzymes in the reaction mixture by partial leaching from the nanoparticles.

When CRL (free and immobilised forms) was used as an enzyme, both optical isomers were formed (see Fig. 3). The ratio of 4-(R)-hydroxycyclopent-2-en-1-(S)-acetate to 4-(S)-hydroxycyclopent-2-en-1-(R)-acetate in the product mixture was around 2:1. Free CRL exhibited the highest % conversion of reactant whereas physically adsorbed CRL performed the lowest conversion. In conclusion, CRL is catalytically less active compared to PFL but suitable for the desymmetrisation of both acetoxy groups of the reactant in an approximately 2:1 ratio.

The reactant *meso*-cyclopent-2-en-1,4-diacetate can forms a tetrahedral intermediate complex with the catalytic triad of the lipase active site (Asp/Glu-His-Ser)[17] and the mono-acetoxy products (see Scheme S1 in ESI†) are hydrolyzed from the active site. The hydrolysed acetoxy group from the diacetoxy reactant remains bound to the serine residue in the active site as an acyl–enzyme intermediate complex. After the acyl–enzyme intermediate is formed, water hydrolyses the acetoxy group, forming acetic acid in the reaction mixture, which enables the acid-catalysed hydrolysis of the mono-acetoxy product **2a** to the dihydroxy by-product **1** when the enzyme is PFL.

In summary, high surface area novel mesoporous core–shell superparamagnetic nanoparticles have been fabricated and used as a support for the immobilisation of two different lipase enzymes *via* recently reported[18] TPRE method. The materials have been tested for one step formation of pharmaceutically important optimal isomers 4-(R)-hydroxycyclopent-2-en-1-(S)-acetate and 4-(S)-hydroxycyclopent-2-en-1-(R)-acetate by enzymatic hydrolysis of *meso*-cyclopent-2-en-1,4-diacetate. It is important to mention that enzyme PFL produced an optically pure enantiomer 4-(R)-hydroxycyclopent-2-en-1-(S)-acetate whereas CRL produced both optical isomers (see Table S2 in ESI† for enatiomeric excess value). Hence the combination of a suitable enzyme and a suitable superparamagnetic support can be an important step for the industrial production of pharmaceutically important optical isomers with a single step separation of expensive enzymes from the reaction mixture by an external magnetic field.

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