Novel Nanocomposites: Hierarchically ordered porous silica for the immobilization of enzyme as bio-catalyst

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ABSTRACT

Hierarchically ordered porous silica with porosity in three different lengths (microporosity: pore diameter < 2nm; mesoporosity: 2nm < pore diameter < 50nm; macroporosity: pore diameter > 50nm) has been synthesized using polystyrene latex as colloidal template to generate macroporosity, pluoronic F127 ($EO_{107}PO_{70}EO_{107}$) as a non-ionic surfactant to generate meso and microporosity in an acidic pH in the presence of tetramethyl orthosilicate as silica source. The calcined material has been functionalized using aminopropyl triethyoxy silane (APTS) in toluene at 50^0C in the presence of surface adsorbed water followed by glutaraldehyde treatment. Lipase has been immobilized by chemical conjugation on the surface of functionalized materials in PBS buffer (pH, 7.4). Hydrolysis of 4-nitrophenyl palmitate has been studied using Lipase immobilized hierarchically ordered porous silica and the results have been compared with lipase immobilized non functionalized materials. The rate of hydrolysis was observed to be increased exponentially with time for both materials, however, functionalized materials exhibited a two fold increase in hydrolysis efficiency without loss of catalytic efficiency after 2nd catalytic cycle.

Keywords: Enzyme catalysis, lipase, hierarchically ordered porous silica, surface functionalization, hydrolysis of ester

1 INTRODUCTION

Complex nanocomposites with porous structures are widespread in nature where hierarchy of pore structure has evolved to provide either strength or a pathway for fluid transport. For instance the structure of calcium phosphate bone is highly porous such that the material is as light as possible but with optimal load bearing properties associated with the distribution of pores and the curvature of the wall structure. The silica exo-skeleton of a diatom is designed both for protection of the single-celled algae and for transport of fluid to the organism within. Nature also gives clues to the optimal structures for transport of high volumes of gases to active sites in the hierarchical pore structure of the lungs. To date, there is a vast literature on synthesizing materials with monodisperse pore sizes at different length scales, e.g. microporous (pore diameter < 2nm), mesoporous (2nm < pore diameter < 50nm) and macroporous (pore diameter > 50nm). However, there is far less on trying to combine these different length scales into one composite material. Sen *et al* reported [1, 2] such hierarchically ordered porous nanocomposites of silica structures using polystyrene latex spheres of defined sizes as a template for macropores, pluoronic tri-block copolymer as a surfactant for meso and microporosity in the presence of a co-solvent, however, the applications of such novel nanocomposites have never been reported. Recently, hierarchically ordered porous materials have been used for the applications such as scaffolds [3] and rechargeable battery [4], however, such nanocomposites have not been exploited as catalytic supports.

Lipase is a well known enzyme used extensively for the bio-catalysis. However, using lipase in solution phase bio-catalysis is not economical as the separation of lipase from the reaction mixtures is not an easy process. One of the important reactions reported using lipase is the hydrolysis of vegetable oil for the production of bio-diesel [5, 6]. Dyal *et al* reported [7] the immobilization of *candida rugosa* on magnetic nanoparticles (maghemite) for the hydrolysis of ester in heterogeneous medium.

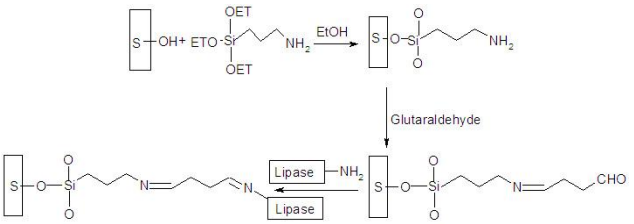
Herein, we report the immobilization of lipase (*candida rugosa*) on novel hierarchically ordered porous silica nanocomposites by chemical conjugation with functionalized surface and their efficiency in the hydrolysis of *p*-nitro phenyl palmitate to p-nitro phenol and palmitic acid as heterogeneous bio-catalysts.

2 EXPERIMENTAL

Hierarchically ordered porous silica materials were synthesized following the previously reported protocol [1, 2]. Non cross linked polystyrene latex spheres were synthesized following the reported protocol using an emulsion polymerization technique [2]. The polystyrene latex spheres were packed into a monolithic structure by centrifugation at 6000 rpm for 1 hr and dried at 60^0C overnight. Polystyrene latex monolith was filled with a gel containing silica (tetramethyl orthosilicate) in an acidic medium (aqueous hydrochloric acid) in the presence of tri-block copolymer F127 and n-butanol. The silica gel filled polystyrene monolith composite was dried at 60^0C overnight. Templates were removed from the composites

by washing with toluene followed by calcinations at 500^{0}C for 8hr in the presence of air.

Lipase (*candida rugosa*) immobilisation was carried out by chemical conjugation and the method has been presented schematically (see reaction scheme 1).

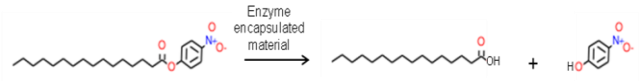


Scheme 1 Reaction scheme for the chemical conjugation of lipase on hierarchically ordered porous silica support (S).

150mg of calcined materials were thoroughly mixed with 10µg deionised water and the wetted materials were functionalised using 5% (w/v) 3-aminopropyl triethoxy silane (APTS) in 10mL toluene at 50^{0}C for 24 hrs in a glass reactor. Surface-adsorbed water was used for the hydrolysis of APTS on the surface of the materials [8]. The surface amine density of functionalised nanocomposite was determined by established colorimetric assay using 4-nitro benzaldehyde [9]. The amine functionalized silica nanocomposites were washed with 1 mL (3 times) of coupling buffer (1× SSC, pH 7.3) for 2 min at RT. 1×SSC buffer was prepared by diluting a stock solution of 20×SSC buffer (175.3 g of NaCl, 88.2 g of sodium citrate, and 1 L of H_2O, pH 7.4) with distilled, deionized water, adjusted to pH 7.4. After removal of the supernatant, 1 mL of a 5% (w/v) glutaraldehyde solution in coupling buffer was added and the reaction mixture was incubated for 3 h with end-over-end rotation at RT. The material was subsequently washed with 1 mL (3 times) of coupling buffer to remove excess glutaraldehyde. The materials were then washed with 1mL (3 times) of PBS buffer (pH 7.2) and stored in PBS buffer before immobilisation of Lipase.

50mg of glutaraldehyde modified silica nanocomposites were treated with 1mL of Lipase solution (2mg/mL) in PBS buffer for 15 hrs with end-over-end rotation at RT. The concentration of Lipase solutions before and after the reaction with silica nanocomposites were measured by Bradford assay [10] using UV visible spectroscopy (absorption at λ_{595nm}). The amount of lipase in the solution was calculated using a calibration curve constructed from a range of standard solutions of lipase prepared separately in PBS buffer reacting with 1.2mL of Bradford reagent and measuring λ_{595nm}. A similar reaction was carried out on calcined silica nanocomposites (non-functionalized) for the immobilisation of lipase.

Hydrolysis of ester (4-nitro phenyl palmitate) was carried out using 15mg support reacting with 1mL of ester solution (3.74 µmol ml^{-1}) prepared in a 1:1 mixture of isopropanol and reagent A (0.0667g Gum Arabic + 12mL of 250mM Tris-HCl buffer, pH 7.8 + 48mL of deionized water + 0.267g of sodium deoxycholate) at 20^{0}C for 4 hrs in 1.5mL Eppendorf tube by end-over-end rotation. The products were collected in different time intervals and measured the concentration of 4-nitrophenol. Scheme 2 presents the hydrolysis of 4-nitro phenyl palmitate to palmitic acid and 4-nitro phenol.



Scheme 2 Reaction scheme for the hydrolysis of 4-nitro phenyl palmitate

The hydrolysis reaction was monitored by measuring the concentration of 4-nitro phenol in the reaction solution. The concentrations of 4-nitrophenol in the reaction mixture at different time interval were determined by using a calibration curve constructed from a range of standard solutions of 4-nitrophenol prepared separately in a solution containing 1:1 mixture of isopropanol and reagent A by measuring the absorbance at λ_{410nm}.

Nanocomposites were washed in 1mL (3 times) buffer solution containing 1:1 mixture of isopropanol and reagent A. The washed materials were further used for the hydrolysis of 4-nitrophenyl palmitate under identical condition as above for testing the catalytic efficiency of lipase immobilized nanocomposites in the 2nd catalytic cycle.

3 RESULTS AND DISCUSSION

Scanning electron micrographs of the hierarchically ordered porous nanocomposites before and after lipase immobilization have been presented in figure 1. Materials exhibited uniform macroporous structure of pore sizes of around 330nm with interconnecting windows of around 100nm. The void structure and interconnecting windows remains free after lipase immobilization indicating that the pore structure remains unchanged which is essential for the transport of reactant molecules into the nanocomposites. The presence of meso and microporosity has been confirmed through low angle powder X-ray diffraction and nitrogen adsorption measurement (data not shown). BET surface area of the calcined materials was measured to be around 210m^2/g. The formation mechanism of such porous silica composites containing macro, meso and microporosity with interconnecting windows of hierarchical ordering has been reported earlier [1, 2].

Surface amine density of the APTS functionalized nanocomposites was measured to be 54nmol/mg by colorimetric assay.

Figure 2A and B presents the calibration curves of lipase in PBS buffer using Bradford reagent (Fig.2A) and 4-nitrophenol in a 1:1 mixture of isopropanol and reagent A (Fig.2B). Calibration curve 2A exhibited a straight line with a positive intercept (regression value of 0.9653) and calibration curve B exhibited straight line passing through

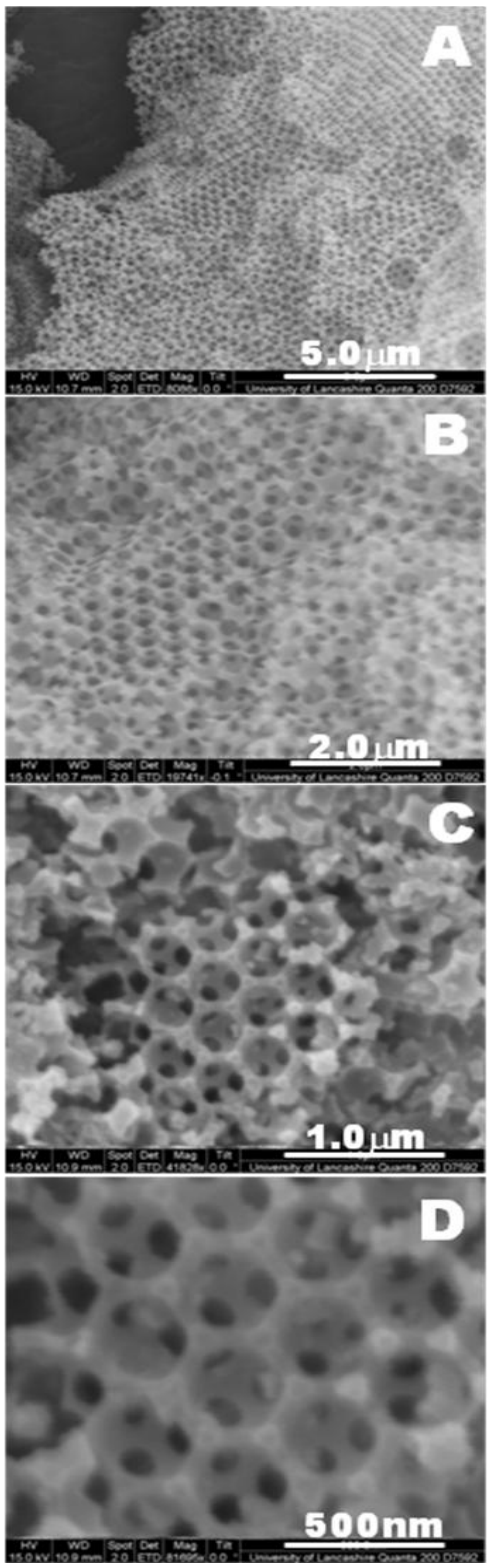


Figure 1 Scanning electron micrographs of hierarchically ordered porous silica. A and B represent calcined nanocomposites before surface functionalization. C and D represent the surface functionalized lipase immobilized nanocomposites

the origin with no intercept (regression value of 0.9853) indicating that the calibration curves are reliable for measuring the concentration of unknown solutions within the concentration ranges presented in figure 2A and 2B.

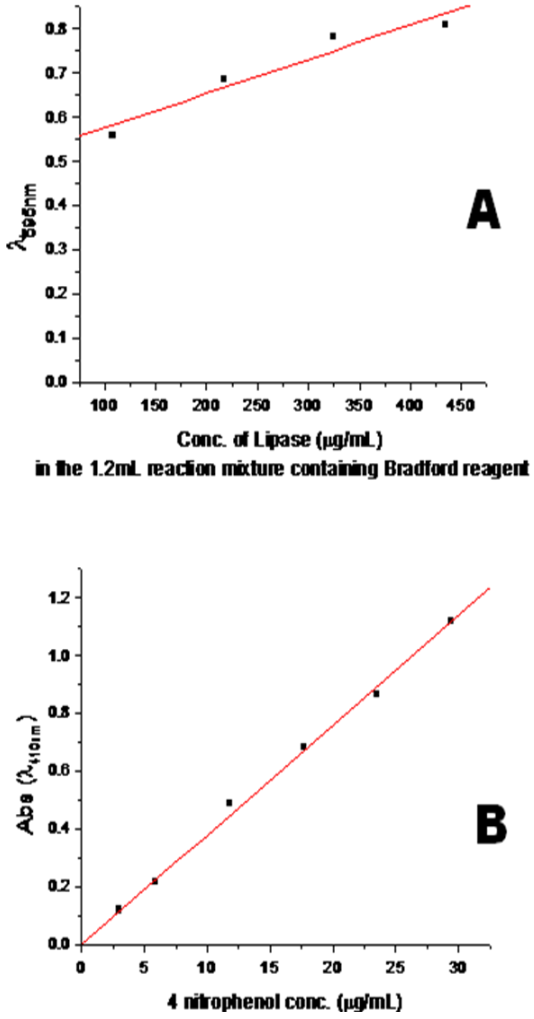


Figure 2 Calibration curves of Lipase in PBS buffer (A) and 4-nitrophenol in 1:1 mixture of isopropanol to reagent A (B)

The lipase concentration on non-functionalized and functionalized nanocomposite has been calculated using calibration curve 2A and the values were 32.3µg/mg and 42.6µg/mg respectively. Immobilization of lipase on functionalized nanocomposites exhibited a change in color from white to pink whereas no color-change observed in non-functionalized nanocomposites.

The kinetics of hydrolysis of 4-nitrophenyl palmitate using lipase immobilized nanocomposites (functionalized and non-functionalized) has been presented in figure 3. The rate of hydrolysis was observed to be increased exponentially with time for lipase immobilized non-functionalized and functionalized nanocomposites.

However, the catalytic efficiency of lipase immobilized functionalized nanocomposites was observed to be two fold higher in value (826 μmol/g of lipase) compared to lipase immobilized non-functionalized nanocomposite (437 μmol/g of lipase). The catalytic efficiency was unaffected on 2nd cycle on lipase immobilized functionalized nanocomposites whereas a six fold reduction was observed in non-functionalized nanocomposites.

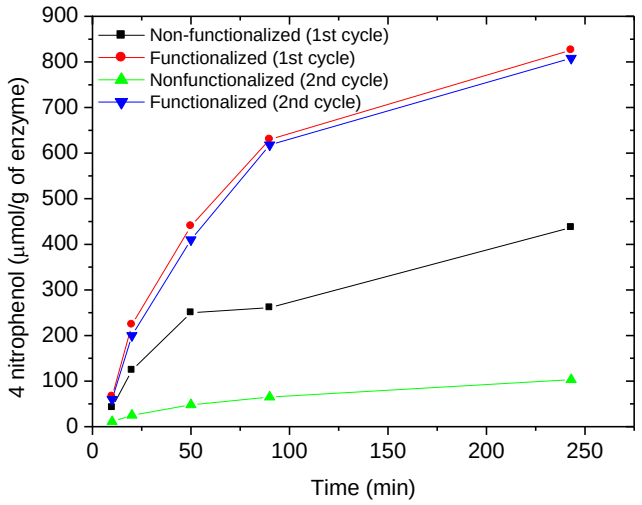


Figure 3 Kinetics of hydrolysis of 4-nitro phenyl palmitate to palmitic acid and 4-nitrophenol

The reason for the loss of catalytic efficiency of lipase immobilized non-functionalized nanocomposites could be due to the leaching of lipase from the nanocomposites into the reaction mixture in the 1st catalytic cycle. Leaching of lipase onto the non-functionalized nanocomposites perhaps due to the loosely bonded lipase to the surface by physical adsorption whereas lipase could be chemically bonded to the functionalized surface through chemical conjugation as presented in scheme 1.

In conclusion, hierarchically ordered porous silica nanocomposites have been used for the immobilization of lipase (*candida rugosa*). Lipase immobilization by chemical conjugation through an intermediate surface functionalization step is important for the fabrication of efficient bio-catalysts. Chemically conjugated novel bio-catalysts reported in this paper could be utilized in other important bio-transformation processes in pharmaceutical industry as a greener route.

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