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Development of effective nanobiocatalytic systems through the immobilization of hydrolases on functionalized carbon-based nanomaterials

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ABSTRACT

In this study we report the use of functionalized carbon-based nanomaterials, such as amine-functionalized graphene oxide (GO) and multi-walled carbon nanotubes (CNTs), as effective immobilization supports for various lipases and esterases of industrial interest. Structural and biochemical characterization have revealed that the curvature of the nanomaterial affect the immobilization yield, the catalytic behavior and the secondary structure of enzymes. Infrared spectroscopy study indicates that the catalytic behavior of the immobilized enzymes is correlated with their α-helical content. Hydrolases exhibit higher esterification activity (up to 20-fold) when immobilized on CNTs compared to GO. The covalently immobilized enzymes exhibited comparable or even higher activity compared to the physically adsorbed ones, while they presented higher operational stability. The enhanced catalytic behavior observed for most of the hydrolases covalently immobilized on amine-functionalized CNTs indicate that these functionalized nanomaterials are suitable for the development of efficient nanobiocatalytic systems.

1. Introduction

Nanobiocatalysis is a rapidly growing research field which refers to the application of enzymes immobilized on nanomaterials (Kim et al., 2008). Recently, the development of nanostructured materials science resulted in a range of nanomaterials with different sizes and shapes, some of which are already used as immobilization matrices (Kim et al., 2008; Pavlidis et al., 2010c). Enzyme immobilization on nanostructured materials presents some advantages over the bulk solid materials, namely the high surface area which can lead to higher enzyme loading, the nanoscale dispersion and the ease of surface functionalization (Kim et al., 2008).

Carbon-based nanomaterials, such as carbon nanotubes (CNTs) and graphene have attracted considerable interest among nanostructured materials for their unique mechanical, thermal and electrical properties as well as for their biocompatibility (Kuchibhatla et al., 2007). These characteristics facilitated their use in electronic devices (Liu et al., 2008), fuel cells (Kauffman and Star, 2010), as carriers for drug delivery (Bianco et al., 2005), or as supports for biomacromolecules immobilization (Cang-Rong and Pastorin, 2009; Gao and Kyratzis, 2008; Pavlidis et al., 2010a,c). Graphene is a single layer of carbon atoms in a honeycomb two-dimensional lattice which has a high specific surface area and can be fabricated from graphite (Park and Ruoff, 2009). CNTs are one-dimensional nanomaterials which can be considered as concentric rolled graphene sheets with a diameter up to 100 nm and length up to a few micrometers; in addition to multi-walled CNTs, single-wall nanotubes can also be prepared (Tasis et al., 2006). The difference in graphene and CNTs curvature results in different properties, such as the higher water dispersability of graphene oxide derivatives compared to the corresponding functionalized CNTs (Kuchibhatla et al., 2007).

The chemical functionalization of nanomaterials is a well-established technique for grafting desirable functional groups onto their surface to obtain nanomaterials with desired properties (Bourlinos et al., 2003; Shim et al., 2002). The surface chemistry of the functionalized nanomaterials can affect their dispersability and interactions with other molecules such as proteins, thus alter the biological

Abbreviations: Bs2, *Bacillus subtilis* esterase 2; CalA, *Pseudozyma* (*Candida*) *antarctica* lipase A; CalB, *Pseudozyma* (*Candida*) *antarctica* lipase B; CI, covalent immobilization; CNTs, carbon nanotubes; CrI, *Candida rugosa* lipase; FT-IR, Fourier transform infrared; GO, graphene oxide; GtI, *Geobacillus thermoleovorans* lipase; NCI, non-covalent immobilization; PestE, *Pyrobaculum calidifontis* esterase; Tween 20, polyethylene glycol sorbitan monolaurate; XPS, X-ray photoelectron spectroscopy.

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activity of the immobilized enzymes (Pavlidis et al., 2010a). For instance, graphene oxide (GO), a graphite derivative decorated with abundantly with epoxide, carboxylic and hydroxyl groups, was recently used as an efficient immobilization matrix, due to its unique chemical and structural properties (Zhang et al., 2010). Although there have been numerous attempts to conjugate enzymes and other biomolecules with carbon-based nanomaterials, there are not enough studies on the influence that the nanomaterial properties (such as composition, morphology, and surface chemistry) have on the structure and function of conjugated proteins. Investigating the structure and function of proteins immobilized on nanomaterials will be crucial for developing a better understanding of protein–nanomaterial interactions and for designing functional protein–nanomaterial conjugates (Asuri et al., 2006a).

In the present work we report the immobilization of several recombinant microbial esterases and lipases of biotechnological interest (Bornscheuer, 2002; Jaeger and Eggert, 2002) on amine-functionalized multi-wall CNTs and graphene oxide, investigating the influence of carbon-based nanomaterials' properties on the immobilization efficiency, function and structure of enzymes. Enzymes were immobilized on both CNTs and GO derivatives via two different methods: (1) physical adsorption and (2) covalent linkage with amine functionalized carbon based nanomaterials. The resulting novel hybrid biocatalysts were characterized by X-ray photoelectron spectroscopy (XPS). The biocatalytic characteristics of the immobilized enzymes (synthesis activity, operational stability) were studied and the conformational changes of enzymes upon immobilization in these carbon-based nanomaterials investigated using Fourier transform infrared (FT-IR) spectroscopy.

2. Methods

2.1. Materials

Lipase from *Candida rugosa* (Crl, 0.084 U/mg) was purchased from Sigma (Germany). Novozyme 735 (lipase A from *Pseudozyma* (*Candida*) *antarctica,* CalA, 194 mU/mg) and Lipozyme CalB L (lipase B from *Pseudozyma* (*Candida*) *antarctica*, CalB, 98 mU/mg) was a generous gift from Novozymes (Denmark). Esterases from *Pyrobaculum calidifontis* (PestE, 0.023 U/mg) and *Bacillus subtilis* (Bs2, 7.1 U/mg) and lipase from *Geobacillus thermoleovorans* (Gtl, 54 mU/mg) were produced by overexpression in *Escherichia coli* (Henke et al., 2002; Hotta et al., 2002; Soliman et al., 2007). One Unit of the aforementioned hydrolases was defined in our laboratory as the amount of enzyme which hydrolyzed 1 μmol of *p*-nitrophenyl butyrate in 1 min, at 30 °C in a phosphate buffer solution (50 mM, pH 7.5). PestE and Gtl were used as crude extracts, while Bs2 was purified using an Äkta Purifier® equipped with a 5 mL Histrap fast flow column (GE Healthcare) and 300 mM imidazole as eluent. Desalting was subsequently done using a Sephadex® G25 column. Multi-wall carbon nanotubes (95% pure, CNTs) were purchased from Aldrich. Graphite was purchased from Fluka. All chemicals used were of analytical grade.

2.2. Enzyme immobilization

The nanomaterials used in our study were functionalized with hexamethylenediamine, according to the procedure described in previous works (Bourlinos et al., 2003; Pavlidis et al., 2010a).

2.2.1. Non-covalent immobilization (NCI)

Hydrolases were non-covalently immobilized on carbon-based nanomaterials by physical adsorption, using a similar procedure as proposed in a previous work (Pavlidis et al., 2010a). In a typical procedure, 5 mg of nanomaterials in 10 mL of phosphate buffer (50 mM, pH 7.5) were sonicated approximately for 30 min. Then 1 mL of enzyme solution containing 0.5–25 mg of enzyme was added and the mixture incubated under stirring for 1 h at 30 °C and then overnight at 4 °C. The bioconjugates were separated by centrifugation from the supernatant (unbound enzyme) and then they were washed twice with buffer solution to remove loosely bound enzyme. The immobilized enzyme was dried over silica gel at 4 °C and was stored at 4 °C until used.

2.2.2. Covalent immobilization (CI)

The covalent immobilization procedure was developed based on the NCI procedure, using glutaraldehyde as cross-linking agent between the enzyme and the amino groups located on the surface of the nanomaterials. In a typical procedure, 5 mg of nanomaterials were sonicated in 9 mL phosphate buffer (50 mM, pH 7.5) for 1 h in the presence of 110 μL Tween 20. After the dispersion of nanomaterials, a quantity of glutaraldehyde was added in order to prepare a 4% (w/w) solution and the volume adjusted to 11 mL. The mixture was incubated at 30 °C for 30 min under stirring. The modified nanomaterials were separated by centrifugation at 6000 rpm for 30 min and washed with buffer solution. Then, 10 mL of buffer solution were added and the nanomaterials sonicated for 1 h in order to form a homogeneous suspension. Enzyme solution (1 mL) containing 0.5–25 mg of protein was added and the mixture was treated as described for the NCI procedure.

2.3. Determination of immobilization yield

The amount of immobilized enzyme was determined by calculating the protein concentration in the supernatant after the immobilization procedure using the bicinchoninic acid (BCA) assay (Smith et al., 1985). All experiments were carried out in triplicate.

2.4. Determination of immobilized enzyme activity

0.5 mg of immobilized lipase or 0.3 mg of lyophilized enzyme were used to catalyze the esterification of caprylic acid (0.1 M) with 1-butanol (0.2 M) in 1 mL of *n*-hexane at 30 °C at 200 rpm. In the case of esterases, 0.5 mg of immobilized enzyme was used to catalyze the transesterification of vinyl acetate (0.1 M) with (*R*,*S*) 1-phenyl ethanol (0.1 M) in 1 mL *n*-hexane at 30 °C and 200 rpm. In all cases aliquots were withdrawn at selected time intervals and were analyzed by gas chromatography. A SPB 5 capillary column (30 m × 0.32 mm × 1.0 μm, Supelco) with helium as carrier gas was used for the analysis on a Shimadzu GC 17A (Japan) device equipped with a flame ionization detector. All experiments were carried out in duplicate.

2.5. FT-IR spectroscopy

IR spectra (32 scans) were measured using a Shimadzu FT-IR 8400 spectrometer (Tokyo, Japan) equipped with a deuterated triglycine sulfate detector. The spectra acquisition, the data analysis of the amide I region and the band assignment were performed as described in previous works (Pavlidis et al., 2010b).

2.6. X-ray photoelectron spectroscopy

Samples were analyzed using a SSX-100 (Surface Science Instruments, UK) photoelectron spectrometer with a monochromatic Al Kα X-ray source ($h\nu$ = 1486.6 eV). The base pressure during the measurement was 2×10^{-10} mbar, and the energy resolution was set to 1.16 eV to minimize measuring time. The photoelectron takeoff angle was 37°. Evaporated gold films on mica served as substrates. The biomaterials produced from the immobilization of enzymes on carbon-based nanomaterials were