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

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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.

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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 (Kaufman 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 Kyratsiz, 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
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 CNFs 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 CNFs 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 Pseudzyma (Candida) antarctica, CaLA, 194 mU/mg) and Lipozyme CaLB L (lipase B from Pseudzyma (Candida) antarctica, CaLB, 98 mU/mg) was a generous gift from Novozymes (Denmark). Esterases from Pyrobaculum caldifontis (PestE, 0.023 U/mg) and Bacillus subtilis (B2, 7.1 U/mg) and lipase from Geobacillus thermoolovorans (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 B2 was purified using an Akta Purifier® equipped with a 5 mL His-trap 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, CNFs) 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/v) 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 (RS) 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 (hv = 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
dispersed in n-hexane and after short stirring, a small drop of the suspension was deposited onto the substrate and left to dry in air. The binding energies of all carbon-based nanocomposites were referenced to the Au 4f_7/2 core level of the substrate (Quintana et al., 2010). Spectral analysis included a background subtraction and peak separation using mixed Gaussian–Lorentzian functions in a least-squares curve-fitting program (Winspec) developed in the LISE laboratory of the Facultés Universitaires Notre-Dame de la Paix, Namur, Belgium. The photoemission peak areas of each element used to estimate the amount of each species on the surface were normalized by the sensitivity factors of each element tabulated for the spectrometer used.

3. Results and discussion

3.1. Hydrolase immobilization on carbon-based nanomaterials

In the present work we investigated the immobilization of various microbial and recombinant hydrolases, widely applied enzymes in biocatalytic processes (Bornscheuer, 2002; Jaeger and Eggert, 2002), on amine-functionalized graphene oxide and multi-walled CNTs. The enzymes used in this study are esterases and lipases with molecular weights varying from 33 to 57 kDa, while their isoelectric point values (pI) were between 4.9 and 7.5. The immobilization procedure was carried out under constant conditions (pH 7.5, 30 °C). At this pH value all hydrolases exhibit high hydrolytic activity (data not shown), while the net charge of the protein molecules is negative (the exception being CalA which has a pI of 7.5).

The immobilization of various hydrolases on both carbon-based nanomaterials was carried out via two different methods, namely physical adsorption and covalent attachment; both are depicted in Scheme I (Supplementary material file). The physical adsorption of enzymes on carbon-based nanomaterials is mostly governed by hydrophobic interactions (Gao and Kyratzis, 2008). For the covalent immobilization of enzymes, the bifunctional reagent glutaraldehyde was used as a cross-linking agent between the amine functionalized nanomaterials and the enzyme, in a similar manner as described for other hybrid biocatalysts (Wu et al., 2009). To avoid the cross-linking of enzyme molecules, the amine-functionalized nanomaterials were first activated with glutaraldehyde. In a following step the free terminal aldehyde groups were cross-linked to amine groups on the enzyme surface through Schiff’s base formation, by incubating the activated support in enzyme solutions. It should be noted that in order to minimize the non-specific adsorption of enzymes onto the support, the covalent immobilization procedure was carried out in the presence of a surfactant compound such as Tween 20. Surfactant molecules have been reported to cover the hydrophobic surface of nanomaterials, rendering them more hydrophilic and thus hinder the non-specific immobilization (Shim et al., 2002). In our study, the hydrolytic activity of CalB immobilized non-covalently on CNTs in the presence of Tween 20 was only 10% compared to that observed when immobilized without surfactant, indicating the protective effect of the surfactant against the non-specific immobilization.

Table 1 shows the efficiency of the non-covalent and covalent immobilization procedures for the immobilization of several hydrolases, using a protein to nanomaterial weight ratio of 3:1. The immobilization yield was calculated from the difference in concentration of the protein in the aqueous phase before and after immobilization. As it can be seen, all hydrolases tested were immobilized successfully to both nanomaterials used, regardless of the immobilization procedure. Immobilization yields up to 55% were achieved, which results in an enzyme loading of 1.65 mg of enzyme per mg of nanomaterial. This enzyme loading value is particularly high compared to the published values for other types of carbon-based nanomaterials (Asuri et al., 2007; Cang-Rong and Pastorin, 2009; Karajanagi et al., 2004). In previous work, we have shown that the immobilization yield could be improved by using a lower enzyme to nanomaterial weight ratio, albeit at the expense of the enzyme loading (Pavlidis et al., 2010a). The high immobilization yield observed for both immobilization procedures may also result from the adsorption of enzyme molecules on top of those previously immobilized (covalently or non-covalently) on the support, in a similar manner as described for other type of nanomaterials (Tzialla et al., 2010).

As it can be seen from Table 1, the use of functionalized CNTs resulted in higher immobilization yields compared to those for graphene oxide derivatives. Taking into consideration that the non-covalent immobilization process relies mostly on hydrophobic interactions between the enzyme and the support (Gao and Kyratzis, 2008), the higher immobilization yield observed in the case of CNTs might result from the higher hydrophobicity of CNTs derivatives compared to the GO ones (data not shown), which results in stronger hydrophobic interactions between CNTs and the enzyme molecules. Moreover, the lower immobilization yield observed with covalent immobilization procedure for both carbon-based nanomaterials used could be due to the limited number of free amine groups on their surface which are available for covalent attachment of enzyme molecules (Cang-Rong and Pastorin, 2009).

3.2. Characterization of the enzyme–nanomaterial bioconjugates using XPS spectroscopy

The functionalized nanomaterials used in the present study were characterized in previous works using transmission electron microscopy, X-ray diffraction, thermogravimetric analysis, Fourier-transform infrared and Raman spectroscopy (Bourlinos et al., 2003; Chen et al., 2006; Pavlidis et al., 2010a). In the present work we used X-ray photoelectron spectroscopy (XPS) in order to investigate how the enzyme molecules are immobilized on the modified CNT and GO surfaces and to distinguish between covalent and non-covalent binding of the enzyme. XPS can provide not only qualitative but also quantitative information on the elemental composition of hybrid systems. The presence of the amide groups of the immobilized enzyme as well as that of nitrogen on the functionalized carbon-based nanostructured materials was established by analysis of the binding energies of the N 1s core level spectra. The knowledge of the chemical state of nitrogen in the final hybrids is of great importance in order to understand the nature of the interactions between the modified CNT or GO surface and the immobilized enzyme. N 1s core level emission spectra of amino-functionalized GO, CNTs and the hybrid systems derived from the non-covalent and the covalent immobilization of the enzyme on the functionalized GO and CNT surfaces are shown in Fig. 1.

The spectra of amino-functionalized CNTs and GO were fitted to the experimental data (constrained by the theoretical intensity ratio) assuming two distinct, chemically shifted N 1s core level emissions, occurring at binding energies of 402.6 eV and 399.5 eV. The former arises from the free protonated amine groups (–NH₃⁺) of hexamethylenediamine (HMD), while the latter is assigned mainly to the nitrogen of the C–NH bond that forms between GO or CNT and HMD during functionalization, but also contains a contribution from non-protonated amine free groups (–NH₂) of HMD (Moulder and Stickle, 1995; Tzialla et al., 2010). A C:N ratio equal to 8.0 was calculated from the relative atomic concentrations of the carbon and nitrogen present in the amine-functionalized graphene oxide, while the corresponding ratio for functionalized CNTs was 9.0 (Table 3), mirroring the high degree of functionalization of these carbon-based nanomaterials.
In the case of the non-covalent immobilization of CalB on the graphene oxide derivative the same N1s photoemission peaks at 402.6 and 399.5 eV were observed, however, their relative intensities were quite different from those of the neat nanomaterial. In fact, the spectral intensity of the peak at 399.5 eV increased significantly with respect to that of the peak at 402.6 eV due to the contribution of the amide bonds of the enzyme at 399.4 eV (Tzialla et al., 2010). This increase of peak at 399.5 eV is presented clearly in Table 2, where the atomic percentages (%) derived from the N 1s photoemission peak areas are presented. Moreover, the C:N ratio decreases upon immobilization of the enzyme, indicating the presence of enzyme molecules on the nanomaterials. The C:S ratio is a better indication of the successful immobilization of the enzyme, as only the enzymes contain sulfur atoms in cysteine and methionine amino acids. As seen in Table 3, higher C:S ratios are observed when the enzyme is covalently bound to CNTs or GO than for the non-covalent immobilization, indicating less enzyme loading, as discussed also in previous section (Table 1). This was expected, as the non-covalently enzyme can be located everywhere on the surface of the nanomaterial, while covalently immobilized molecules can attach only to specific sites.

The covalent immobilization of CalB on these amine-functionalized nanomaterials is testified to by the appearance of an additional N1s component at 399.0 eV binding energy in the XPS spectra, which is attributed to the carbon–nitrogen double bond (\(-\mathrm{C}=\mathrm{N}\)) (Moulder and Stickle, 1995; Pietrzak, 2009). The intensity of the peak at 402.6 eV is more depleted than for the non-covalently immobilized enzyme, indicating that the free amine groups on the functionalized nanomaterials and on the enzyme molecule are used for the formation of the covalent bond.

### 3.3. Activity of immobilized hydrolases

The enzyme to nanomaterial weight ratio is a crucial factor that could affect the catalytic behavior of immobilized enzymes (Pavlidis et al., 2010a). The effect of this ratio on the catalytic activity of covalently and non-covalently immobilized enzymes was investigated using, as model reaction, the synthesis of butyl caprylate in anhydrous \(n\)-hexane. As seen in Fig. 2, the effect of the enzyme to nanomaterial weight ratio seems to follow a pseudo-saturation curve, which is in accordance to that previously reported for soybean peroxidase immobilized on single-walled
Moreover, in all cases studied, the activity of the enzyme was significantly higher when immobilized on functionalized CNTs compared to that observed when graphene oxide derivative was used as immobilization support, which seems to correlate to the enzyme loading (Table 1) and the curvature of the nanomaterials, as will be discussed later (Table 4).

It is interesting to note that under non-saturation conditions (low enzyme–nanomaterials weight ratio), the covalently immobilized enzyme exhibited higher synthetic activity than the non-covalently bound enzyme. A possible explanation is that the enzyme molecule tries to maximize its contact with the hydrophobic surface when it is non-covalently immobilized on the nanomaterial’s surface, which could lead to undesirable conformational changes and hence loss of activity (Tzialla et al., 2010). The higher enzyme activity observed in NCI procedure when higher enzyme concentration was used could be attributed to a possible adsorption of the new enzymes molecules on top of the previously adsorbed ones rather than on the support surface, as described before.

It should be noted that using enzyme to nanomaterial weight ratio values higher than 3 no significant effect on the immobilized enzyme activity was observed, independently on the immobilization procedure used (Fig. 2). At this weight ratio, the effect of the immobilization procedure on the synthetic activity of various hydrolases was investigated in anhydrous media. As shown in Table 4, the immobilized enzymes exhibited significant higher activity than the non-immobilized ones. CalB exhibited the highest synthetic activity among the lipases tested, namely up to 20-fold higher than free enzyme, while Gtl covalently immobilized on CNTs exhibited more than 75-fold higher activity than when used as free enzyme. Similar behavior was also observed for the synthesis of butyl laurate (data not shown). On the other hand, esterase Bs2 did not exhibit any activity after immobilization (except after non-covalent immobilization in GO derivative), although it was successfully immobilized on both CNTs and GO (as discussed in Section 3.1). It must be noted that the incubation of Bs2 esterase in aqueous solution with both types of nanomaterials (at a concentration up to 25 μg/mL) led to fast deactivation of the esterase. Such deactivation was not observed with the other hydrolases used, which indicates that the effect of the carbon-based nanomaterials on the enzyme catalytic behavior as well as on the effectiveness of the immobilization process is highly enzyme dependent. Covalently immobilized enzymes exhibited comparable activity with non-specifically immobilized enzyme, and in some cases (Crl, Gtl and PestE) even higher. The beneficial effect of functionalized nanomaterials used on most hydrolases activity, as described in Table 4, is independent on the concentration of the substrates used in a range of 0.05–0.20 M. This result differs to that reported for the immobilization of amyloglucosidase on CNTs, where the observed activity was significantly lower for the covalently immobilized enzyme than for the non-covalently attached one (Cang-Rong and Pastorin, 2009).

Notably CNT-immobilized enzymes exhibited up to 10-fold higher activity than GO-immobilized ones, as described also for CalB in Fig. 2. It is expected that the size and shape of...
nanomaterials affect the interactions with proteins and therefore the structure and catalytic behavior of the immobilized enzymes (Cang-Rong and Pastorin, 2009; Mu et al., 2008; Vijayaraj et al., 2010). The higher activity observed when CNTs were used as immobilization matrix could be attributed to their curvature (Mu et al., 2008). The flatter surface of the GO derivative may trigger significant conformational changes in enzyme molecules and thus result in lower activity biomaterials, as discussed also in Section 3.5. Similar enhanced synthetic activity of the immobilized hydrolytic enzymes on CNTs was also observed for other polar or less polar organic solvents (such as acetonitrile and tert-butanol, respectively) and imidazolium-based ionic liquids (data not shown).

3.4. Structural studies

The conformational changes of the hydrolases upon immobilization on both carbon-based nanomaterials were investigated by FT-IR spectroscopy. The analysis of the Amide I band at approximately 1700–1600 cm⁻¹ (mainly due to the C=O stretching vibration) makes it possible to obtain information on the effect of the immobilization on the secondary structure of the protein (Secundo et al., 2007). The Amide I band consists of several overlapping components that are assigned to different secondary structure elements. These individual components were identified from the second derivative spectra (Natalello et al., 2005). Suggestively, the FT-IR spectra of the Amide I region of free CalA and immobilized on carbon-based nanomaterials is provided in Supplementary material file. The α-helix content is a good indicator of the proper folding of the enzyme. The loss of this secondary structure element usually leads to enzyme inactivation, although an intact α-helix structure is no guarantee for an active enzyme. Table 5 presents the α-helix content of various hydrolases before and after covalent and non-covalent immobilization on amine-functionalized CNTs and GO.

As it can be seen in Table 5, all hydrolases underwent some conformational changes upon immobilization on both nanomaterials, in agreement with what was observed for other supports (Asuri et al., 2007; Cang-Rong and Pastorin, 2009; Karajanagi et al., 2004; Tzialla et al., 2010). The α-helix content of all covalently-immobilized enzymes on both nanomaterials increased compared to that of free enzymes. More specifically, the α-helix content of CalA increased when immobilized both covalently or non-covalently onto CNTs. In this case, the CalA exhibited increased esterification activity compared to the free enzyme (Table 4), which indicates a correlation between the increase in α-helix content and the enhanced catalytic activity. A similar correlation between α-helix content and activity was also observed for CalB when covalently immobilized on both nanomaterials, which is in agreement with recently reports on enzymes immobilized on various hydrophobic organically modified silica glasses (Mena et al., 2008).

The α-helical content of CalA and CalB was depleted when the lipases were immobilized on graphene oxide compared to what was observed when CNTs were used as immobilization support. These changes in secondary structure observed after enzyme immobilization on GO is mirrored in a lower catalytic activity, compared to that observed for immobilization on CNTs, in a similar manner as discussed before. The different effects of CNTs and GO on the enzymes activities and structures could be related to specific interactions of enzymes with these nanomaterials (Kim et al., 2008), as indicated by XRD results in Section 3.2. The positive effect on the lipases’ catalytic behavior by CNTs could be also related by the increased curvature of CNTs compared to graphene oxide in a similar manner as described by Asuri et al. for protein immobilization on single-walled CNTs (Asuri et al., 2006). Namely, the increased curvature of CNTs could probably contribute to a reduction of detrimental interactions between immobilized protein molecules given that these molecules are further apart in this case; this in turn leads to increased enzyme stability on CNTs compared to that on flat graphene oxide.

However, the fact that esterase Bs2 exhibited synthetic activity only when non-covalently immobilized on the graphene oxide derivative (in this case the α-helix content was highest as shown in Table 5), indicates that the catalytic behavior of the enzymes as well as their conformational changes occurring upon immobilization depend not only on the nature of nanomaterial used and the immobilization procedure, but also on the specific enzyme.

3.5. Operational stability of immobilized CalB

The viability of an industrial application based on immobilized enzymes depends on whether the enzyme can be regenerated and consequently whether the biocatalyst can be reused. The operational stability of hydrolases covalently and non-covalently immobilized on carbon-based nanomaterials was investigated. Enzymes immobilized on GO and CNTs derivatives were used at 60 °C in anhydrous n-hexane and the activity was determined as described in Section 2.4. Each reaction cycle lasted 24 h. Then, the immobilized enzyme was washed three times with n-hexane, and reused. Suggestively, the operational stability of CalB for butyl caprylate synthesis is presented in Fig. 3.

Fig. 3 demonstrates that all immobilized enzyme preparations retain more than 50% of their activity after five repeated uses (120 h of total operation time) at 60 °C. The covalent immobilization procedure has a significant stabilizing effect on CalB for both

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**Table 4**

Initial reaction rate (mmol h⁻¹ mg⁻¹ enzyme) of the esterification reaction for lipases and the transesterification reaction for esterases. The weight ratio of enzyme to nanomaterial in the immobilization procedure was 3:1 (standard deviation was less than 5% in all cases).

<table>
<thead>
<tr>
<th>Preparation</th>
<th>CalA</th>
<th>CalB</th>
<th>CrI</th>
<th>Grl</th>
<th>Esterases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-immobilized</td>
<td>0.33</td>
<td>9.33</td>
<td>2.0</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>CNTs</td>
<td>1.15</td>
<td>191.40</td>
<td>3.20</td>
<td>11.70</td>
<td>0.00</td>
</tr>
<tr>
<td>Graphene oxide</td>
<td>0.15</td>
<td>15.45</td>
<td>0.33</td>
<td>6.47</td>
<td>0.38</td>
</tr>
</tbody>
</table>

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**Table 5**

Quantitative estimation (%) of the α-helical content of free lipases and lipases immobilized on amine functionalized CNTs and GO, as estimated from the analysis the amide I region of FT-IR spectra (standard deviation was less than 5% in all cases).

<table>
<thead>
<tr>
<th>Preparation</th>
<th>CalA</th>
<th>CalB</th>
<th>Bs2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyophilized</td>
<td>25</td>
<td>27</td>
<td>22</td>
</tr>
<tr>
<td>CNT-NCI</td>
<td>30</td>
<td>20</td>
<td>26</td>
</tr>
<tr>
<td>CNT-CI</td>
<td>35</td>
<td>36</td>
<td>27</td>
</tr>
<tr>
<td>GO-NCI</td>
<td>22</td>
<td>18</td>
<td>31</td>
</tr>
<tr>
<td>GO-CI</td>
<td>26</td>
<td>28</td>
<td>30</td>
</tr>
</tbody>
</table>
nanomaterial used. Covalently immobilized CalB on CNTs retains about 60% of its initial activity after 14 uses (336 h of total operation time), while the non-covalently immobilized enzyme lost more than 90% of its activity after 9 uses. A similar behavior was observed for other hydrolases used in the present work (data not shown). The stabilizing effect of carbon based nanomaterials as immobilization matrix observed here is in accordance with what was previously observed for various enzymes covalently immobilized on oxidized CNTs (Asuri et al., 2006a).

It is interesting to note that CalB when non-covalently immobilized on GO derivative is more stable than when physically adsorbed to CNTs, despite the fact that it exhibited lower catalytic activity (Table 4). This could be due to stronger hydrophobic interactions of the enzyme with the GO derivative surface, which could prevent enzyme leakage, as described previously (Section 3.3).

4. Conclusions

In this work, we report the development of innovative nanobiocatalytic systems through the immobilization of several recombinant hydrolytic enzymes of significant industrial interest on carbon-based nanomaterials such as amine-functionalized multi-wall carbon nanotubes and graphene oxide. Biochemical and structural studies indicate that the immobilization approach and the curvature of the nanomaterials significantly affect the immobilization efficiency, the esterification activity, the secondary structure and the operational stability of immobilized enzymes. The enhanced catalytic behavior observed for most of the hydrosols covalently immobilized on amine-functionalized CNTs indicate that these functionalized nanomaterials are suitable for the development of efficient nanobiocatalytic systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.11.007.

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