Organofunctionalized silica gel as a support for lipase

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A B S T R A C T
This work described the preparation of silica gel modified with cyanuric chloride. The modified silica gel was tested for the ability to immobilize Burkholderia cepacia lipase. Contact times ranging from 4 to 24 h were investigated to determine the optimal lipase immobilization time. Following immobilization, the enzyme activity was assessed by the hydrolysis of p-nitrophenylpalmitate (pNPP). Elemental analysis data revealed that 0.4 mmol of cyanuric chloride were anchored per gram of support, demonstrating the successful incorporation of the triazine molecule onto the silica surface. The tests of reusability and storage indicated that the enzyme-modified silica-gel (immobilization at 24 h) was more stable under reaction conditions than the other systems. The activity assays indicated high rates of enzymatic formation of p-nitrophenol (p-NP), demonstrating a maximum activity retention of 87%.

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1. Introduction
The use of lipases (triacylglycerol acylhydrolases) as biocatalysts in organic reactions is of great interest in the processing of oils and fats because these enzymes exhibit the ability to catalyze the hydrolysis of esters, liberating fatty acids and glycerol, as well as the reverse reaction, that is, ester synthesis [1–3]. Recently, these enzymes have been investigated in biocatalysis, particularly in the synthesis of pharmaceuticals, to obtain pure enantiomer compounds and to produce biodiesel by transesterification [1,3–5].

The ability of lipases to catalyze a variety of reactions is a result of their regioselectivity, enantioselectivity, and chemoselectivity as well as with their stability in organic solvents [4–6]. The use of lipolytic enzymes in bioprocesses is particularly advantageous when the enzymes are immobilized onto a solid support, enabling the facile recovery and reuse of the immobilized enzyme over several cycles of the reaction without a significant loss of biological activity. In addition, immobilized lipases may show superior activity compared to the free enzyme, including greater thermal and pH stability [7–9].

Lipases can be immobilized on a variety of supports, including materials that are organic (cellulose, chitin, chitosan), inorganic (silica, clay, alumina), hydrophilic or hydrophobic, and by several methods including ion adsorption, covalent binding, crosslinking, encapsulation, or immobilization in membranes [1,10–12]. Both the support and the immobilization method are critical in determining the thermal stability and the mechanical and chemical properties of the resulting biocatalyst.

The cost of immobilization must also be considered [13]. Accordingly, inorganic supports exhibit numerous advantages compared with organic supports, due to their superior mechanical and thermal properties [14–16]. In terms of the immobilization method, covalent bonding provides a more effective interaction between the enzyme and the support, leading to a more chemically and thermally stable enzyme interaction and minimizing desorption of the enzyme during the biocatalytic process [17–19].

Due to the diverse conditions of biocatalysis reactions, it is important to consider a variety of solid supports, immobilization methods and reaction parameters to maximize the stability of the enzyme and to minimize losses in catalytic activity.

Previously, both natural and synthetic media have been used as matrices for lipase immobilization to obtain a biocatalyst with high catalytic efficiency that can be recovered and reused for several reaction cycles.

In this study, the synthesis and characterization of silica gel modified with cyanuric chloride was investigated. This silica gel was used to immobilize a commercial lipase from Burkholderia cepacia. The catalytic behavior of the immobilized enzyme was evaluated by the hydrolysis of p-nitrophenylpalmitate (p-NPP).

2. Materials and methods
2.1. Materials
The following materials were used: chromatographic silica gel (Sigma-Aldrich) with a 70–230 mesh particle size, a median diameter of 60 Å, and a pore volume of 0.75 m³ g⁻¹; (3-aminopropyl) trimethoxysilane 97% (Aldrich); cyanuric chloride 99% (Acros...
Organics); Amano Lipase PS from *B. cepacia* (Sigma-Aldrich); bovine serum albumin BSA (Sigma); protein Biuret reagent (Ambresco®); and Folin–Ciocalteau’s phenol reagent (Sigma–Aldrich). The chemicals 1,4-dioxane, ethanol 99%, and isopropanol 98% were used as solvents and were obtained from Vetec. Toluene was purchased from Synth. The substrates p-nitrophenylpalmitate (Sigma–Aldrich) and gum arabic (Aldrich) were used for the activity assays.

2.2. Functionalization of the silica gel support

The silica gel was functionalized using an organosilane containing an amino functional group as a modifying agent. Prior to the chemical modification of the inorganic matrix, the silica was activated by heating at 150 °C under vacuum for 12 h. This procedure was necessary to remove water from the silica surface, allowing the silanol groups to react with the silylating agent. Once activated, the silica (Sil-At) reacted with 3-aminopropyltrimethoxysilane (15.0 mL, 86.0 mmol) in a reflux of toluene (150.0 mL) under mechanical agitation and a nitrogen atmosphere at 120 °C for 72 h. The resulting aminopropylsilica (Sil-N) was washed sequentially with toluene, ethanol, and deionized water.

2.3. Reaction of cyanuric chloride with aminopropylsilica

The organosilane-modified silica gel (Sil-N) was allowed to react with cyanuric chloride (7.5 g, 40.7 mmol) under mechanical stirring in 150.0 mL of a mixture comprising 1,4-dioxane/toluene in a 4:1 ratio at 12–20 °C for 12 h. This synthesis was based on previous procedure [20]. The material was then filtered and washed with toluene and acetone. The resulting functionalized solid was termed Sil-NCC.

2.4. Characterization of modified silicas

The functionalized silicas were characterized by the elemental analysis of CHN and Cl (Fisons Instruments Elemental Analyzer, Model EA – 1110 CHNS-O). Infrared spectroscopy ( Nicolet 380 Spectrophotometer Thermo) was performed using KBr pellets and a spectral range of 4000 to 400 cm$^{-1}$. Thermogravimetry (DTG-60H Shimadzu Thermal balance) measurements were collected under a nitrogen atmosphere with a flow rate of 50 mL min$^{-1}$ and a heating rate of 10 °C min$^{-1}$.

2.5. Immobilization of lipase on silica modified with cyanuric chloride

The immobilization of lipase from *B. cepacia* was performed at varying time intervals (4, 8, 12, 16, 20, and 24 h) to investigate the influence of contact time on the amount of immobilized protein and the enzyme activity. A 10.0 mL volume of enzyme solution (2.00 mg mL$^{-1}$) dissolved in phosphate buffer (66.0 mmol L$^{-1}$, pH 7.2) was added to 150 mg of the functionalized support. The system was allowed to react under stirring at 150 rpm and 25 °C. Following immobilization, the solids were washed thrice with 5.0 mL of immobilization buffer to remove any weakly adsorbed enzyme residues.

2.6. Determination of lipase loading

The amount of protein present in the supernatant solutions (following immobilization) and the solutions leached with the enzyme (following washing of the support) was spectrophotometrically determined by the method of Lowry (1951) [21] using bovine serum albumin (BSA) as a standard. The samples containing the enzyme were measured by absorbance at 660 nm using a UV/VIS spectrophotometer (TECNAL Spectrophotometer 2000 UV/VIS) against a pH 7.2 blank buffer solution without enzymes. The amount of protein bound to the support was calculated as the difference between the amount of protein dissolved in the solution prior to immobilization and the amount of protein remaining in the filtered solutions and the buffer wash following immobilization. The mass of protein retained per gram of support was calculated using Eq. (1).

\[
p = \frac{C_i V_i - (C_s V_s - C_f V_f)}{m_s}
\]

where \(p\) represents the amount of bound protein (mg g$^{-1}$ support), \(C_i\) is the initial concentration of the protein (mg mL$^{-1}$), \(C_s\) is the concentration of the protein (mg mL$^{-1}$) present in the filtered solutions, \(C_f\) (mg mL$^{-1}$) is the concentration of the leached protein, \(V_i\) (mL) is the volume of the solution used to immobilize the enzyme, \(V_s\) (mL) is the volume of the supernatant solution, \(V_f\) is the volume (mL) of the support washing solution, and \(m_s\) is the support mass. All experiments were performed in triplicate, and an average was calculated from the experimental data.

2.7. Enzyme activity

The enzyme activities of free and immobilized lipase were spectrophotometrically determined using the method reported by Winkler and Buckmann [22]. The procedure involved the hydrolysis of p-nitrophenylpalmitate in the presence of free (200 mg mL$^{-1}$) or immobilized enzyme (30.0 mg) by incubation in a pre-heated medium (37 °C, pH 8.0) for 15 min. The reaction releases the yellow compound p-nitrophenol (pNP), which exhibits an absorption peak at 410 nm. Samples were measured against a blank containing a pH 8.0 buffer without enzymes. The retention of enzyme activity was calculated according to Eq. 2, using the molar extinction coefficient \(c = 15 \text{kcm}^2\text{mol}^{-1}\)·cm$^{-1}$.

\[
\%U_r = \frac{U_{\exp} \times 100}{U_i}
\]

Here, \(U_i\) is the recovered activity, \(U_{\exp}\) is the experimentally observed activity from the immobilized enzyme, and \(U_i\) is the theoretical maximum activity.

2.8. Reusability

Reusability was evaluated in terms of the recovery of activity over the course of pNPP hydrolysis experiments. For this study, we used the derived enzyme, which exhibited superior immobilization efficiency and retention of enzymatic activity. The immobilized biocatalysts were subjected to five consecutive cycles under the same reaction conditions used to assess the hydrolytic activity of the substrate. Following each reaction cycle, the samples were washed consecutively with isopropanol alcohol and pH 7.2 phosphate buffer.

2.9. Storage stability

The lifetime of the immobilized biocatalysts was evaluated by determining the residual enzyme activity under the reaction conditions established in Section 2.7 after 30 days of storage at 4 °C without the use of solvent. Following the storage period, the enzymes were employed in the hydrolysis of p-NPP for five consecutive batches. After each reaction interval, the support was washed thrice with 5.0 mL of pH 7.2 phosphate buffer. The residual activity was determined as described in Section 2.7.

2.10. Statistical treatment

To minimize the propagation of errors, all glass instruments used in the determinations were calibrated. For each step of the procedure,
the same instruments were used across all samples to minimize systematic errors.

The statistical uncertainty, obtained by standard deviation, has been reported with the data. For each set of measurements, the standard deviation (SD) was calculated using Eq. (3).

\[
SD = \sqrt{\frac{\sum (X_i - \bar{X})^2}{n-1}}.
\]

Where \( X \) is the value, \( \bar{X} \) is the mean value, and \( n \) is the number of measurements.

3. Results and discussion

3.1. Characterization of the modified silica gel support

3.1.1. Infrared spectroscopy

Infrared spectra were collected for the solids Sil-At (activated silica), Sil-N, and Sil-NCC as presented in Fig. 1. The spectrum of Sil-At exhibits the typical bands of the inorganic polymer silica gel. In the region of 3500 cm\(^{-1}\), a broad band attributed to the O–H stretching frequency is observed, and at 1650 cm\(^{-1}\), a peak deformation is observed; both of these features are related to physically adsorbed water molecules. In the region of low energy, the spectrum also exhibits a broad band between 1100 and 1000 cm\(^{-1}\) assigned to the stretching vibrations of Si–O and Si–O–Si, which are structural characteristics of the inorganic silica [23].

For Sil-N, the spectrum exhibits the same characteristic bands as Sil-At, indicating the preservation of the structural integrity of the inorganic matrix. However, a decrease in the intensity of the absorption approximately 3500 cm\(^{-1}\) (\(\nu\)O–H) and weak absorption in the region of 2830 cm\(^{-1}\) are also observed, suggesting the presence of C–H stretching [23,24] and indicating the successful functionalization of Sil-At with the silylating agent (3-aminopropyl)trimethoxysilane. For Sil-NCC, the same peaks and absorption bands present in Sil-N are observed; in addition, new bands appear between 1750 cm\(^{-1}\) and 1500 cm\(^{-1}\), that can be attributed to the stretching vibrations of the cyanuric chloride triazine ring. These absorption bands (1750 and 1446 cm\(^{-1}\)) indicate that Sil-N was chemically modified by the anchoring of the triazine compound on its surface, thereby resulting in the Sil-N-CC material.

3.1.2. Thermal analysis

Thermogravimetric curves (TG) can provide important information about the chemical modification of the surface of the inorganic matrix, as well as the thermal stability of the modified materials. The TG curves of the materials Sil-At, Sil-N, and Sil-NCC are presented in Fig. 2.

The curve obtained for Sil-At exhibited only a single step mass loss up to approximately 110 °C, corresponding to the loss of water molecules adsorbed on the surface of the material. For Sil-N and Sil-NCC, the curves detected a lower water loss compared with the inorganic matrix, suggesting an increase in the hydrophobicity of these materials. For Sil-N, the second mass loss event (above ~300 °C) was attributed to the gradual decomposition of the anchored organosilane molecules. Finally, the Sil-NCC curve exhibited a progressive loss of mass over a higher temperature range, possibly due to the presence of longer carbon chain organic molecules anchored to the surface. Thus, for temperatures above 300 °C, the mass losses observed in Sil-NCC were attributed to the simultaneous decomposition of the silane molecule and the cyanuric chloride anchored to the support. These observations suggest the effectiveness of the chemical modification of the inorganic matrix.

3.1.3. Elemental analysis

The carbon, hydrogen, nitrogen, and chlorine content of Sil-N and Sil-NCC were also analyzed as displayed in Table 1. The values (mmol/g of the support) obtained by elemental analysis express the quantity of organic groups anchored to each surface. In the first reaction step, the functionalization of the inorganic matrix involved the covalent incorporation of organosilanes onto the silica surface. Elemental analysis revealed that 1.3 mmol of nitrogen per gram of solid were anchored to the inorganic matrix, suggesting the effectiveness of the reaction and, consequently, the introduction of propylamino groups onto the surface.

In the subsequent step, the presence of amino groups allowed nucleophilic attack by reactive chlorine atoms of the triazine molecule. The effectiveness of this reaction was evidenced by an increase in the nitrogen content of 0.9 mmol g\(^{-1}\) for Sil-NCC compared with Sil-N as well as the anchoring of 1.2 mmol g\(^{-1}\) of chlorine to the surface.

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**Fig. 1.** Infrared spectra of the solids (a) Sil-At, (b) Sil-N, and (c) Sil-NCC.

**Fig. 2.** Thermogravimetric curves from (a) Sil-At, (b) Sil-N, and (c) Sil-NCC.
3.2. Immobilization of lipase

3.2.1. Immobilization of lipase on silica modified with cyanuric chloride

Studies report that the immobilization of enzymes on a solid support may involve weak and/or strong interactions depending on the method and the type of support used for immobilization [25]. Thus, enzymes can be adsorbed by non-covalent interactions such as ionic and hydrophobic interactions, van der Waals forces, and hydrogen bonding, but covalent interactions can also occur if the support contains a reactive species capable of binding to side chains of the enzyme [26,27].

Studying the kinetics of enzyme immobilization is an important process, as a long period of immobilization is often required to obtain an effective interaction between the enzyme and the support that is capable of conferring favorable chemical and thermal stability to the immobilized biocatalyst. Consequently, we investigated the influence of contact time between the B. cepacia lipase and the activated support on the amount of enzyme linked to the support. The immobilization of enzymes on Sil-NCC was investigated using reaction time intervals of 4, 8, 12, 16, 20, and 24 h. The results are depicted in Fig. 3 and listed in Table 2.

The immobilization measurements revealed a progressive increase in the retention of protein per gram of support with increasing contact duration, with the highest protein immobilization observed at 24 h. During this period, approximately 116 mg of protein per gram of support were immobilized, reflecting the need for a long contact time to achieve efficient enzyme immobilization. These data suggest that shorter contact intervals may result in the predominance of weak interactions such as van der Waals forces, hydrogen bonding, and hydrophobic interactions between the enzyme and the support. This was confirmed by the desorption of the immobilized enzymes upon leaching with the immobilization buffer. The enzyme concentrations in the washing solutions were determined and subtracted from the initial concentration of the enzyme available for immobilization, as described by Eq. (1).

The increased interaction time may have favored the formation of more effective links between the chemical groups of the support and the reactive groups of the enzyme, allowing the amount of immobilized enzyme to increase significantly. These results can be attributed to the activation of the support with the coupling reagent cyanuric chloride, as the triazine molecule exhibits a docking mechanism with the reactive chlorine atoms that can promote the nucleophilic substitution reaction between the triazine ring and the side chain residues of the enzyme. The reaction sequence, including the reaction of silica with silane, the incorporation of the triazine molecule, and the covalent attack by the amino groups of the enzyme, is depicted in Scheme 1.

### Table 2

<table>
<thead>
<tr>
<th>Support</th>
<th>Immobilization time intervals (h)</th>
<th>Enzyme load&lt;sup&gt;a&lt;/sup&gt; (mg g&lt;sup&gt;−1&lt;/sup&gt; support)</th>
<th>Activity&lt;sup&gt;b&lt;/sup&gt; (μmol min&lt;sup&gt;−1&lt;/sup&gt; g&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>Retention activity, U&lt;sub&gt;1&lt;/sub&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sil-Lip1</td>
<td>4</td>
<td>25.3 ± 0.5</td>
<td>1843 ± 1</td>
<td>46.2</td>
</tr>
<tr>
<td>Sil-Lip2</td>
<td>8</td>
<td>28.0 ± 0.3</td>
<td>2338 ± 1</td>
<td>58.6</td>
</tr>
<tr>
<td>Sil-Lip3</td>
<td>12</td>
<td>30.7 ± 0.3</td>
<td>2800 ± 3</td>
<td>70.2</td>
</tr>
<tr>
<td>Sil-Lip4</td>
<td>16</td>
<td>57.3 ± 0.4</td>
<td>2815 ± 2</td>
<td>70.5</td>
</tr>
<tr>
<td>Sil-Lip5</td>
<td>20</td>
<td>86.6 ± 0.5</td>
<td>2852 ± 2</td>
<td>71.5</td>
</tr>
<tr>
<td>Sil-Lip6</td>
<td>24</td>
<td>116.0 ± 0.5</td>
<td>2910 ± 1</td>
<td>72.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Quantity of enzyme for immobilization: 133 mg g<sup>−1</sup> support.

<sup>b</sup> Theoretical maximum activity: 3990 U g<sup>−1</sup> support.

The plots in Fig. 4 demonstrate that the amount of immobilized enzyme was greatly influenced by the contact time between the enzyme and the support when the reaction time was greater than 12 h. However, the protein loading amount did not exert a significant influence on the rate of conversion of p-nitrophenol during the hydrolysis of p-NPP. The enzymatic activity was nearly constant for the immobilized lipases Sil-Lip3, Sil-Lip4, Sil-Lip5, and Sil-Lip6, despite the sequentially increasing quantities of immobilized enzyme. These results suggest that the immobilized lipase reaches a maximum level of catalytic activity, beyond which increases in the concentration of the enzyme will not necessarily affect the rate of enzymatic hydrolysis. This behavior is typically attributed to the steric hindrances that can occur upon the formation of multiple layers of enzymes [28], leading to protein denaturing or diffusion limitations that hinder the access of the substrate to the active sites of the immobilized enzymes [29,30].

In addition, it is worth noting that the behavior of an enzyme in a heterogeneous medium may differ from that in homogeneous systems, even without the influence of diffusional restrictions. It is therefore reasonable to assume that the enzyme molecules are immobilized at random, resulting in heterogeneity in the enzyme orientation and behavior. This hypothesis is also reasonable in light of the chemical nature of the amino acids on the surfaces of enzymes, as the chemical groups of amino acid residues near the active center can react with the active surface of the support [31]. Thus, it is possible that some of the immobilized enzymes are poorly oriented or even denatured due to the conformational changes of the active site during the immobilization process or catalysis [32,33]. These phenomena would result in lower enzymatic activity, even for large quantities of immobilized enzyme.
3.2.3. Reusability

The effect of immobilization on the reusability of lipases was evaluated by measuring the enzymatic activity of immobilized derivatives after five consecutive p-NPP hydrolysis cycles. The results are depicted in Fig. 5. It can be observed that the immobilized lipases exhibited a loss of activity after each reaction cycle, although Sil-Lip6 displayed greater stability compared with the other systems, maintaining increased activity under the hydrolysis assay conditions. A slight loss of enzyme activity is expected, as the immobilized enzymes can undergo desorption upon washing with the immobilization solution in each reaction cycle. Because the loss of activity was less pronounced for the Sil-Lip6 system, it is likely that the enzyme molecules in this system formed more effective links with the active surface of the support.

Scheme 1. Silylation of silica gel with 3-aminopropyltrimethoxysilane and the subsequent reaction with cyanuric chloride followed by the covalent reaction with the lipolytic enzyme.

3.2.4. Storage stability

The storage stability determines the lifetime of an immobilized enzyme. In this study, the immobilized enzymes were stored for 30 days and subsequently used for the hydrolysis of p-NPP for five consecutive reaction cycles.

The immobilized lipases exhibited high rates of retention of the enzymatic activity during the first reaction cycle. However, after the

Scheme 2. Catalytic reaction for the hydrolysis of p-nitrophenyl/palmitate.

Fig. 4. Percentage retention of the catalytic activity (U g⁻¹ support) and the amount of immobilized enzyme (mg g⁻¹ support) as a function of immobilization time.
successive batches, the enzyme exhibited the same loss of activity observed in the freshly immobilized enzyme, with Sil-Lip6 once again exhibiting slightly greater stability compared with the other systems, as depicted in Fig. 6. Moreover, the immobilized enzymes remained stable throughout the fourth and fifth reaction cycles for Sil-Lip3, Sil-Lip4 and Sil-Lip6. These results indicate that the immobilized lipases can maintain their biological activity under the tested storage conditions. This stability may be due to the formation of effective links between the reactive groups of the enzyme molecules and the surface of the support.

4. Conclusions

Silica surfaces were activated using coupling reagent cyanuric chloride and covalently modified with lipase from \textit{B. cepacia}. Immobilization studies demonstrated the high affinity of the lipolytic enzyme for the silica surface. By studying the kinetics of immobilization, the load of the enzyme on the solid support was found to depend on contact time, with a time of 24 h providing the maximum enzyme loading (116 mg g\(^{-1}\) support). However, it was observed that beyond a specific amount of protein loading, further increases in the amount of immobilized enzyme did not lead to improvements in the catalytic activity. In terms of stability, the Sil-Lip6 system demonstrated excellent potential for recovery and reuse in hydrolysis reactions, exhibiting superior stability compared with the other systems that were studied.

Finally, the propylamino silica activated with cyanuric chloride efficiently immobilized lipase from \textit{B. cepacia} under the conditions employed in this study, demonstrating its potential as a catalyst for the hydrolysis of the ester \textit{p}-nitrophenylpalmitate.

Acknowledgments

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References


Fig. 5. Effect of the reuse of the immobilized lipases (\(\bullet\) Sil-Lip3, (\(\bigtriangleup\) Sil-Lip4, (\(\bigstar\) Sil-Lip5, and (\(\bigcirc\) Sil-Lip6) on the retenion of activity (RA) with respect to the rate of formation of \(p\)-nitrophenol (1 U = mol \(p\)-NP min\(^{-1}\)). The immobilized enzyme was stored at 4–8 °C and washed with phosphate buffer (0.67 M, pH 7.2) after each cycle.

Fig. 6. Effect of storage time on the residual activity (RA) of the immobilized lipases (Sil-Lip3, Sil-Lip4, Sil-Lip5, and Sil-Lip6). The immobilized enzymes were stored for 30 days at 4–8 °C and reused after washing with phosphate buffer (0.67 M, pH 7.2) following each cycle. Enzymatic activity: 1 U = 1 µmol \(p\)-NP min\(^{-1}\).