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Controlled manipulation of enzyme specificity through immobilization-induced flexibility constraints

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ABSTRACT

It is thought that during immobilization enzymes, as dynamic biomolecules, may become distorted and this may alter their catalytic properties. However, the effects of different immobilization strategies on enzyme rigidity or flexibility and their consequences in specificity and stereochemistry at large scale has not been yet clearly evaluated and understood. This was here investigated by using as model an ester hydrolase, isolated from a bacterium inhabiting a karstic lake, with broad substrate spectrum (72 esters being converted; 61.5 U mg^{-1} for glyceryl tripropionate) but initially non-enantiospecific. We found that the enzyme (7 nm × 4.4 nm × 4.2 nm) could be efficiently ionic exchanged inside the pores (9.3 nm under dry conditions) of amino-functionalized ordered mesoporous material (NH_2-SBA-15), achieving a protein load of 48 mg g^{-1}, and a specific activity of 4.5 ± 0.1 U mg^{-1}. When the enzyme was site-directed immobilized through His interaction with an immobilized cationon the surface of two types of magnetic micro-particles through hexahistidine-tags, protein loads up to 10.2 μg g^{-1} and specific activities of up to 29.9 ± 0.3 U mg^{-1}, were obtained. We found that ionically exchanged enzyme inside pores of NH_2-SBA-15 drastically narrowed the substrate range (17 esters), to an extent much higher than ionically exchanged enzyme on the surface of magnetic micro-particles (up to 61 esters). This is attributed to differences in surface chemistry, particle size, and substrate accessibility to the active site tunnel. Our results also suggested, for the first time, that immobilization of enzymes in pores of similar size may alter the enzyme structures and produce enzyme active centers with different configuration which promote stereochemical conversions in a manner different to those arising from surface immobilization, where the strength of the ionic exchange also has an influence. This was shown by demonstrating that when the enzyme was introduced inside pores with a diameter (under dry conditions) slightly higher than that of the enzyme crystal structure a biocatalyst enantiospecific for ethyl (*R*)-4-chloro-3-hydroxybutyrate was produced, a feature not found when using wider pores. By contrast, immobilization on the surface of ferromagnetic microparticles produced selective biocatalysts for methyl (*S*)-(+)-mandelate or methyl (*S*)-lactate depending on the functionalization. This study illustrates the benefits of extensive analysis of the substrate spectra to better understand the effects of different immobilization strategies on enzyme flexibility/rigidity, as well as substrate specificity and stereochemistry. Our results will help to design tunable materials and interfaces for a controlled manipulation of specificity and to transform non-enantiospecific enzymes into stereo-chemically substrate promiscuous biocatalysts capable of converting multiple chiral molecules.

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1. Introduction

Enzymes are delicate organic catalysts that need to be stabilized to survive a range of challenging conditions typically used in industrial processes. Multiple immobilization methods and materials have been successfully employed to generate stabilized biocatalysts [1–4], but the most efficient immobilization protocol and materials may be selected aiming at a balance between activity, specificity, stability, and costs [5]. Besides most used carriers such as (epoxy)acrylic resins and agarose and widely used nanoparticles such as chitosan and nanoflowers [6], outstanding recent examples for enzyme immobilization and stabilization included biomimetic silica supports [7], mesostructured onion-like silica [8], hybrid macroporous foams synthesized via an integrative chemistry synthetic pathway [9], superparamagnetic silica/iron oxide nanocomposites with mesostructured porosity [10], maghemite (γ-Fe_2O_3) nanoparticles functionalized with a reactive multifunctional polymer [11], dendronized polymer and mesoporous silica nanoparticles [12], carbon nanotubes and polymers [13], epoxy-activated carriers [14], borosilicate [15], hematite nanoparticles [16], phyto-inspired silica nanowires [17], and materials with bioinspired coatings [18], to cite some. These studies exemplify the large interest in designing new and more efficient carriers for enzyme immobilization, the comparative analysis of which at large, in relation to their effect in enzyme substrate specificity (including enantiospecificity), may deserve further interest.

A major advance in the last decade has been the development of enzyme encapsulation in ordered mesoporous materials, because of their high specific surface area and pore volume, their highly uniform and tunable pore sizes, and the possibility to create micropores interconnecting mesopores and to control their morphology; they are also thermally, mechanically and chemically stable [5,19–23]. Various proteins have been successfully immobilized on mesoporous materials, including, non-catalytic proteins, oxidoreductases, hydrolases, lyases and isomerases [5,24–41]. Enzymes immobilized in confined macromolecular environments where the surface and volume of the confined environment can be controlled, are suggested to constitute optimal enzymatic nanoreactors [19,42,43]. Their surface can be grafted with different functionalities [23,17,18], a property particularly interesting to allow, for example, the ionic exchange enzyme encapsulation in amino-functionalized ordered mesoporous materials. This development led to an increased enzyme loading (up to 187 mg g^{-1} of catalyst) by simply adjusting synthetic conditions of the siliceous material for the obtaining of pores large enough to permit enzyme entrance and diffusion through the pore channels [17,18]. As example, mesoporous materials with pore diameters as low as 3–10 nm and also tailored between 3–30 nm have been synthesized [for examples see ref. 19,23,25,28,29,31], and enzymes with sizes such as 6.1 nm × 5.0 nm × 4.9 nm [20] have been immobilized in the pores. In these ordered structures the enzyme is immobilized by electrostatic interactions with limited diffusional restrictions and low enzyme modification, typically occurring after covalent binding or crosslinking [44]. This ion exchange of the enzyme on the support, was demonstrated to minimize leaching and maximize activity and stability of the biocatalyst [17,18].

Compared to free enzyme in solution, immobilization on a surface often hinders the free movement of the enzyme, although it is believed that the enzyme is positioned in an environment where the inherent flexibility is high [45]. The confinement in the pores of mesoporous materials is also thought to hinder the free movement of enzyme, but to what extent the differences in movement and flexibility affects enzyme properties remains to be elucidated. In this direction, it is only supposed that larger substrates may be preferably hydrolyzed by enzymes immobilized on the external surface or at the entrance of the channels of mesoporous materials because of substrate diffusional and enzyme orientation issues, as it was demonstrated by modelling predictions [46] and also using a lipase when tested with small (tributyrin) and large (triolein) substrates [47]. However, a general overview about how enzyme immobilization inside pores affects substrate specificity compared to the free enzyme and surface-immobilized enzyme remains to be established. Indeed, diverse surface immobilization methods have been shown to preserve activity level and, most importantly, to even create enantiospecific enzymes such as lipases [for example see ref. 48–50]. These, and other studies, have been performed using a restricted set of molecules and a comparative analysis at large is lacking.

Here, we examine the substrate range and enantiospecificity of a target enzyme by using as supports, an amino-functionalized ordered mesoporous material (NH_2-SBA-15) allowing non-covalent enzyme immobilization inside pores and two types of magnetic microparticles for non-covalent surface immobilization. Particularly, agarose-coated ferromagnetic core-shell microparticles with a nitrilotetracetic acid (NTA) tetradentate ligand, and polyvinyl alcohol microparticles embedded with magnetite and grafted with an iminodiacetic acid (IDA) tridentate ligand. Both particles were Ni^{2+}-activated and thus can be used for purification and immobilization given the affinity for hexahistidine-tags. As model enzyme, a serine ester hydrolase isolated from the metagenomic DNA of microbial communities inhabiting a karstic lake [51], referred to as EH1, with a typical α/β hydrolase fold as a model. Its structure was recently solved (PDB code 5JD4) [51,52], and in a recent study it was identified as one of the ester hydrolases with broader substrate spectrum among a total of 147 when tested with a set of 96 chemically and structurally different esters [51]. The active site (catalytic triad: Ser161, Asp256 and His286; oxyanion hole: Gly88, Gly89 and Gly90) supports the hydrolysis of a broad range of 72 esters, with vinyl butyrate (200.7 ± 0.4 U mg^{-1}) and phenyl propionate (198.7 ± 0.9 U mg^{-1}) serving as the best substrates (Table S1). Being highly promiscuous in terms of substrate scope, EH1 is not enantiospecific; thus the apparent enantiospecificity (E_{app}) factor calculated as the ratio of specific activities for 14 chiral esters when pure stereoisomers were tested separately [53] was below 25, a value above which ester hydrolases begin to have practical value [54]. Based on these considerations, EH1 may be then considered as an ester hydrolase with prominent substrate promiscuity but with limited practical use due to the low stereospecificity. Because substrate promiscuity and enantiospecificity are two appreciated properties when combined in a single biocatalyst, which is rare in nature within esterases [54], we evaluated whether both properties can be tailored by employing three immobilization strategies.

To the best of our knowledge, detailed analysis of the substrate spectrum and interpretation of the data provides, for the first time, new insights into the contribution of chemical, physical, and flexibility constrains to the catalytic capacity of biocatalysts prepared by these immobilization methods. We would like to highlight that the effect of immobilization on enzyme flexibility has been previously studied, for example, by increasing the number of bonds by which the enzyme is linked to the surface of carriers [48–50]. It is though that this increases enzyme rigidity and in turn has a consequence in promoting enzyme stability and activity when tested over a specific set of substrates [48–50]. The novelty of this study is that through an analysis of the substrate specificity with a very broad spectrum of molecules, and 3 different immobilization techniques, we were able to demonstrate that each polymeric carrier induced different flexibility and rigidity constraints to the enzyme with distinct effects on specificity and, most importantly, enantiospecificity, which can be also controlled.

2. Experimental section

2.1. Chemicals and EH1 protein source

All chemicals used for enzymatic tests were of the purest grade available and were purchased from sources described elsewhere [51]. The isolation of the enzyme EH1, available in the expression plasmid pET46 Ek/LIC and *Escherichia coli* BL21 as a host, was reported previously [51,52].