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Advanced characterization of immobilized enzymes as heterogeneous biocatalysts

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ABSTRACT

Like in chemical catalysis, there is a clear trend in biocatalysis to carry out synthetic transformations at the manufacturing scale heterogeneously catalyzed. Recycling of insoluble catalysts is simplified, and continuous reactor development thus promoted. Heterogeneous biocatalysis usually involves enzymes immobilized on mesoporous solid supports that offer a large internal surface area. Unraveling enzyme behavior under the confinement of a solid surface and its effect on the catalytic reaction in heterogeneous environment present longstanding core problems of biocatalysis with immobilized enzymes. Progress in deepening the mechanistic understanding of heterogeneous biocatalytic conversions is often restrained by severe limitations in methodology applicable to a direct characterization of solid-supported enzymes. Here we highlight recent evidence from the analysis of protein distribution on porous solid support using microscopic imaging methods with spatiotemporal resolution capability. We also show advance in the use of spectroscopic methods for the analysis of protein conformation on solid support. Methods of direct characterization of activity and stability of immobilized enzymes as heterogeneous biocatalysts are described and their important roles in promoting rational biocatalyst design as well as optimization and control of heterogeneously catalyzed processes are emphasized.

1. Immobilized enzymes: great catalysts for chemical process development

Modern chemical synthesis strives for synthetic routes that are selective, atom- and step-efficient and inherently safe. Use of enzymes as catalysts potentially enables all of these tasks to be achieved at once [1–3]. Bio-catalysis has therefore been identified, and already serves, as key enabling technology for chemical synthesis at the industrial process scale [3–6]. Like in chemical catalysis, there is the fundamental choice between homogeneous and heterogeneous biocatalysis [4,7–9]. Homogeneous biocatalysis involves enzymes dissolved in aqueous liquid phase. Heterogeneous biocatalysis involves enzymes in a water-insoluble (solid) form [9–11]. Clear trend to prefer heterogeneously bio-catalyzed reactions in current industrial production processes is recognized, in consequence of two main advantages. Firstly, separation and thus recycling of the catalyst are simplified when enzymes are present insoluble. Secondly, continuous biocatalytic process development is supported ideally [4,7,8]. Benefits of continuous processing for high-quality chemicals manufacturing can thus be exploited fully. Different principles of heterogeneous biocatalyst preparation have been described in almost countless varieties [9]. However, the principle most widely used by virtue of overall practical effect is immobilization of an initially soluble enzyme on a mesoporous solid support [10,12–14]. The support is usually selected to offer a high internal surface area accessible to and chemically suitable for the enzyme to become attached physically, chemically or often both [9–11,15–19].

Good choice of an immobilization requires that considerations from the various underlying disciplines, including protein chemistry and enzymology, materials and surface sciences, and reaction engineering, are all integrated adequately. Designing an

Abbreviations: AFM, atomic force microscopy; CD, circular dichroism; CLSM, confocal laser scanning microscopy; DLR, dual life-time referencing; DRIFT, diffuse reflectance infrared Fourier transform spectroscopy; FESEM, field emission scanning electron microscopy; FTIR, Fourier transformed infrared spectroscopy; IR, infrared spectroscopy; MIRS, mid-infrared spectroscopy; NIRS, near-infrared spectroscopy; NMR, nuclear magnetic resonance spectroscopy; PM-IRRAS, polarization-modulated infrared reflexion absorption spectroscopy; QCM, quartz crystal microbalance; SECM, scanning electrochemical microscopy; SIRMS, synchrotron infrared microspectroscopy; SPR, surface plasmon resonance; STEM, scanning transmission electron microscopy; TEM, transmission electron microscopy; XPS, X-ray photoelectron spectroscopy.

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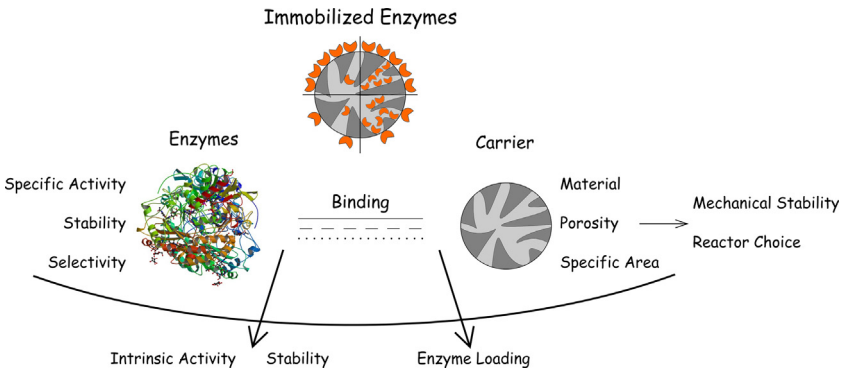

Fig. 1. Enzyme immobilization in porous carriers is shown. Influence of parameters related to the support, the enzyme and the mode of enzyme-on-surface deposition on observable catalytic properties.

immobilization is complicated not only by the multidisciplinary nature of the problem, but also because relevant effects occur at vastly different length scales in the nanometer (molecular) to millimeter range [10,15,16,20]. Therefore, this exacerbates the selection of suitable methodology for monitoring of the immobilization process and for characterization of the final enzyme immobilizate. Preparation of immobilized enzymes for biocatalytic use needs to be practical and cost-effective. Highly demanded features of the final immobilizate are adequate loading of enzyme activity relative to the unit mass of support as well as high-enough stability of both the enzyme activity and the support under conditions of use [9–11]. Fig. 1 illustrates important parameters of the support, the enzyme and the mode of enzyme-on-surface deposition and it also shows how systematic variation of these parameters affects outcome of the immobilization regarding criteria of activity and stability.

2. Understanding the behavior of enzymes immobilized on solid support

Characterization of solid-supported immobilized enzymes nearly always involves comparison to the free enzyme in terms of specific activity and stability [9,18,19]. Note: specific activity is typically expressed as a reaction rate/unit mass of the protein preparation used. Commonly used dimension is μmol/(min × mg). Less often, the reaction rate is related to the moles of enzyme or enzyme active site in which case the specific activity has the dimension of a turnover frequency (1/min). The immobilizate is normally less active than the free enzyme, with a percentage of retained activity anywhere between usually 5 to around 80% [9,18,19]. There are two principal factors underlying the effect, of which one is the direct consequence of structural distortions in the enzyme resulting from attachment to the solid surface and another is indirect consequence of enzymatic reaction taking place in a heterogeneous environment [16,17,19,21] (Scheme 1a). Enzyme stability is often positively affected by the immobilization [18,19]. The stabilizing effect is dramatic in certain cases, but well-grounded mechanistic interpretations based on conclusive direct evidence are typically not available [18,19]. Unraveling enzyme behavior under the confinement of a solid surface and its effect on the catalytic reaction in porous support present longstanding core problems of biocatalysis with immobilized enzymes [16,21]. Progress in deepening the mechanistic understanding of heterogeneous biocatalytic conversions is often restrained severely by limitations in methodology applicable to a direct characterization of solid-supported enzymes [20,22]. Therefore, despite substantial efforts over decades, perfecting an enzyme immobilizate to a specific activity approaching that of the free enzyme (or another target value) remains an elusive task. Lacking direct evidence, optimization of immobilized enzymes in regard to activity and stability is mostly addressed empirically and is not well predictable in its outcome [17,17,20,21].

Fig. 2 depicts a productive cycle of characterization of immobilized enzymes that moves from initial evaluation of basic parameters to advanced direct examinations at different levels of resolution under test conditions as well as in real (*in operando*) studies. Note: the term *in operando* as herein used is distinguished from the mere in situ in implying realistic conditions of immobilized biocatalyst application. Suggestion from Fig. 2 is that running through the cycle in an iterative manner would constitute a paradigmatic approach of systematic development and optimization of immobilized enzymes. This review describes where we stand in the efforts to close the development cycle for heterogeneous biocatalysts. Opportunities from an emerging set of imaging methods with spatiotemporal resolution capabilities are emphasized and research needs to overcome current limitations are identified. An optimal design of heterogeneous biocatalysts would be built on evidence from advanced characterization of biocatalysts (Scheme 1b), which ideally provided a comprehensive and detailed understanding of the relationship between reaction kinetics and structural features of the catalyst elucidated at the relevant length scale.

3. Enzyme loading in high capacity and high quality for immobilized biocatalyst preparation

For practical and economic use, heterogeneous biocatalysts are required to exhibit a specific activity that is as high as possible. Unlike specific activity of the enzyme as soluble or immobilized preparation (see above), the specific activity of the heterogeneous biocatalyst is normally related to the unit mass of solid support and its dimension therefore is μmol/(min × $g_{support}$). In the first instance, the specific activity is determined by the quantity of enzyme mass that can be loaded onto the support and maximizing this amount presents a clear strategy for catalyst optimization [4,9,23]. The internal surface area of the immobilization support accessible to the enzyme via pores of suitable geometry is evidently of high importance [10,11]. The interaction between enzyme and solid surface is another key parameter [9,17,19]. It provides a lot of room for optimization regarding protein-binding capacity through molecular engineering of the enzyme, the surface or both. Balance between surface hydrophilicity and hydrophobicity, surface charge, functional/reactive surface group density and distribution are all critical aspects in the selection of a suitable support [10,15,17]. The presence of covalent attachment sites on the surface is another significant feature to be considered. Covalent coupling is usually not a main factor of the protein binding capacity but it ensures the protein attachment to become quasi-permanent. Instead of modifying the support, targeted modification of the surface of the enzyme presents a fully complementary possibility of