Review

Special Issue: Celebrating 30 years of biotechnology

Shine a light on immobilized enzymes: real-time sensing in solid supported biocatalysts

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Enzyme immobilization on solid supports has been key to biotransformation development. Although technologies for immobilization have largely reached maturity, the resulting biocatalysts are not well understood mechanistically. One limitation is that their internal environment is usually inferred from external data. Therefore, biological consequences of the immobilization remain masked by physical effects of mass transfer, obstructing further development. Work reviewed herein shows that opto-chemical sensing performed directly within the solid support enables the biocatalyst's internal environment to be uncovered quantitatively and in real time. Non-invasive methods of intraparticle pH and O_2 determination are presented, and their use as process analytical tools for development of heterogeneous biocatalysts is described. Method diversification to other analytes remains a challenging task for the future.

Immobilized enzymes for biocatalytic transformations

Biocatalysis has become a key enabling technology for modern organic synthesis [1–3]. A growing number of industrial processes for production of fine chemicals and pharmaceuticals rely on the splendid catalytic prowess of enzymes, providing an unsurpassed combination of selectivity characteristics and reaction rate acceleration [4–9]. Biocatalysis promotes inherently safe and sustainable process development, thus contributing to fulfillment of green chemistry principles at large [5,6,10,11]. There is the general trend in process chemistry that solid supported insoluble catalysts are favored over soluble catalysts, mainly because heterogeneous catalysis is ideal for processes to be performed continuously [7,12]. Applying enzymes, this usually means that immobilization on a solid support, typically a porous carrier, is included as key unit operation in the biocatalytic process [12–14]. Whole cells are sometimes regarded as a cost-efficient alternative due to their being insoluble without the requirement for extra 'heterogenization' [7,15]. However, unfavorable mechanical properties of whole cells (e.g., high compressibility, low structural resistance to shear) severely restrict the available design options for continuous process development [7,15]. Therefore, we recognize little overlap in the main applications of immobilized enzymes and those of whole cells. Multistep biotransformations requiring concerted and spatially confined action of two or more enzymes are often effectively realized using whole cells, however, typically in batch or fed-batch operation and without catalyst re-use [16–18]. Continuous bioprocessing therefore remains the domain of solid supported immobilized enzymes [7,12,13,19].

Enzyme immobilization is a technology underpinned from various disciplines, including material science, protein chemistry, enzymology, and biochemical engineering in particular [20–25]. As a result of extensive research over many decades, its basic principles have been worked out in great detail, and a variety of practical and thoroughly validated protocols exist [13]. Kinetic theory for action of immobilized enzymes is also well advanced [7,19,26]. The overall impression of enzyme immobilization therefore might be one of a rather mature technology. However, when confronted with the problem of a new immobilization, one quickly realizes that the development still involves a large amount of empirical work whereas rational design has only a limited role in it [21,24]. Optimizations are performed in a multidimensional parameter space where key influencing factors, such as chemistry of enzyme-carrier coupling, are difficult to be implemented in today's accelerated-throughput design-of-experiments studies [12,27–29]. One important difficulty roots in the limited understanding of the behavior of enzymes at solid surfaces [20,21]. A second major complication is traced back to mass transfer effects in immobilized enzymes, which include film and pore diffusion as well as interphase partitioning [7,19,26]. Reaction conditions (e.g., substrate concentrations, pH) inside the enzyme carrier therefore often differ substantially from the conditions in bulk liquid (Figure 1) [7,19,26,30,31]. Characterization of the internal environment of enzyme immobilizates is, however, strongly limited by availability of adequate analytical methods. Therefore, internal conditions are almost always inferred from external data (Figure 1), and a global enzyme effectiveness parameter η is employed to compare activities of the immobilized and the free enzyme. The lumped nature

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Keywords: biocatalysis; immobilization; solid support; internal sensing and monitoring; intraparticle pH and O_2 concentration; process analytical technology.

 0167-7799/$ – see front matter http://dx.doi.org/10.1016/j.tibtech.2013.01.004

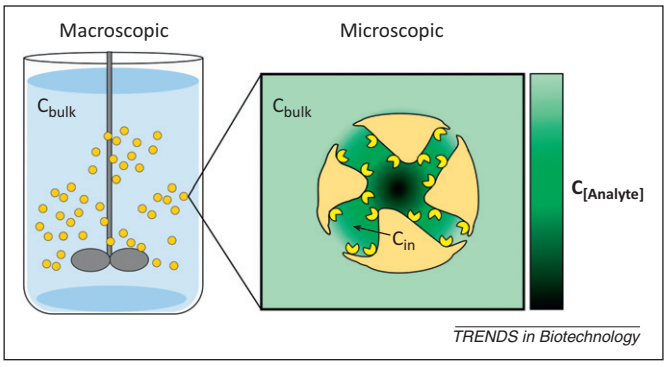

Figure 1. Formation of concentration gradients in porous enzyme carriers. The analyte's concentration in the well-mixed liquid bulk (C_{bulk}) often differs from the concentration inside the carrier (C_{in}). For substrates, C_{bulk} may be higher than C_{in}, whereas for products, the situation may be reversed. Space-averaged measurement of C_{in} allows determination of an overall concentration difference. Spatially resolved measurements are needed to obtain the full internal profile of C_{in}, represented by the light-to-dark green scale.

of η precludes determination of the intrinsic activity of the immobilized enzyme, unmasked from the influence of mass transfer [7,19,26,31]. This presents a major restriction to targeted immobilization development, which requires that biochemical effects of the immobilization be clearly separated from the physical ones.

Recent studies have made significant progress in the characterization of enzyme immobilizates, showing that opto-chemical sensing performed directly within the solid support enables the biocatalyst's internal environment to be analyzed quantitatively and in real time. Generic methods for the determination of intraparticle pH and O_2 concentration have been developed. The two analytes are of general importance in the field because many enzymatic reactions consume or release protons [7–9], and O_2 is a universal cosubstrate for a broad class of oxygenase and oxidase enzymes [32–35]. We will show here that not only do internal data facilitate uncovering the principal factor(s) limiting the effectiveness of the immobilized enzyme and therefore inform design of an optimally active biocatalyst on solid support, but they also suggest novel strategies for monitoring and control of bioprocesses catalyzed by immobilized enzymes. In this paper, we review the process analytical tools developed so far for immobilized enzymes and describe their successful use in different application studies. We also summarize important future tasks, such as method expansion to other analytes, and consider the relevance of 'internal sensing' in biotechnology as a whole.

Opto-chemical sensing in enzyme immobilizates

Principles of opto-chemical sensing of O_2 and pH

The dissolved O_2 concentration and the pH are key measurement and control parameters in numerous biotechnology applications [36–38]. Their determination *in situ* often involves well-established and robust electrochemical sensing procedures, applying amperometric Clark electrodes for O_2, and ion-selective glass electrodes for pH [39,40]. Newer electrochemical methods are based on chemical field effect transistors [39]. However, the relatively large size of electrochemical sensors limits their application at the microscale, and there is the fundamental disadvantage of active consumption of O_2 during the measurement. Opto-chemical sensors, which have become commercially available during the last decade, present an attractive alternative [41]. They consist of luminescence indicator molecules immobilized in an analyte-permeable polymer layer, which can be deposited on a fiber tip or on the walls of a transparent reaction vessel. These sensor layers can be readout contactless from the outside, using optical fibers connected to an opto-electronic measurement device (Figure 2). Opto-chemical pH and O_2 sensors are available in various formats such as layers or spots, fiber-optic (micro)sensors, and sensor particles in micro- or nanometer size [42,43]. These formats allow for a non-invasive flexible application at suitable measurement positions, and for an analytic readout at a microscopic scale. Optical pH sensors have a narrower operating range (~3 pH units) than electrochemical sensors. However, biotechnology applications are usually controlled within a relatively small pH range anyway, and there is the additional advantage of high sensitivity. Cross-sensitivity of pH sensing to ionic strength has recently been eliminated [44].

Opto-chemical O_2 sensors operate according to the principle of dynamic quenching of the phosphorescence of an indicator dye. The quenching affects both intensity and lifetime of the phosphorescence, whereby lifetimes are typically in the range 1–100 μs. The measurement of lifetime is generally superior because it is an intrinsically referenced parameter, which contrary to measurement of intensity is not affected by scattering, reflection, drifts in the opto-electronic setup, and inhomogeneous distribution or bleaching of the indicator. Lifetime can be determined in the time domain, but also in the frequency domain [45–47] (Box 1). Optical pH sensors change their absorption or emission properties depending on the indicator dye's protonation state. Fluorescent pH indicators exhibit lifetimes below <100 ns, requiring a higher degree of instrument sophistication as compared to O_2 sensors. However, a straightforward approach to pH measurement involves ratiometric data collection where the ratio of emission intensity at two wavelengths is determined [48]. Alternatively, a method called dual lifetime referencing (DLR) is applied to convert the intensity signal into a referenced signal, either a phase shift or a time-dependent parameter, by adding a phosphorescent reference dye to the sensing layer [49,50] (Box 1). Using slight modification of the procedure applied for single analyte determination, dual sensing of pH and O_2 has also been reported [46].

Immobilization of luminescence dyes in solid support

Opto-chemical sensors are already in wide use for determination of pH and O_2 in solution. However, carrying out the same measurements directly inside a porous carrier is not possible using the available equipment. Therefore, carriers need to be made internally responsive to pH and O_2, and an attractive possibility to do so is through incorporation of suitable luminescence dyes. Direct conjugation of the enzyme used to a pH-sensitive luminophor (e.g., fluorescein thioisocyanate, FITC) has been explored [51,52]. Alteration of enzyme function is however a potential drawback. Therefore, labeling of carrier instead of enzyme has also been tested. Examples include entrapment in alginate beads [53,54], covalent incorporation into poly(ethylene glycol) (PEG) microparticles [55] and membranes [56], and ionic