Smart enzyme immobilization in microstructured reactors

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ABSTRACT

There has been much recent interest in the application of microstructured flow reactors as tools of biocatalytic process research and development. Continuous biotransformations are often best performed using a heterogeneous biocatalyst, obtained through immobilization of the applied enzyme preparation on the surface of the reactor’s internal (micro)structure. Smart integration of enzyme immobilization in the design and operation of microstructured reactors has presented a considerable challenge for development. The immobilization should be stable, but also readily reversible to allow re-use of the microstructured element. We describe recent advances in the field of microscale enzyme immobilization and summarize recent applications of immobilized enzyme microreactors for biocatalytic synthesis.

KEYWORDS

Biocatalysis; microreactor; enzyme immobilization; biocatalytic flow chemistry; coated microchannel.

BIOTRANSFORMATIONS IN MICROSTRUCTURED REACTORS

Microreaction technology has become a powerful engineering tool for chemical process research and development. Microstructured flow reactors, in short microreactors, are now applied to perform an array of chemical transformations, replacing traditionally used systems such as the stirred tank reactor operated in batch mode (1). The use of highly automated microreactors was shown to provide the process development with interesting new opportunities (2, 3). First of all, reaction screening and optimization can be performed in a fast manner at controlled conditions and at low material input. Secondly, due to their excellent mass and heat transfer characteristics, microreactors potentially enable the process development with an expanded window of operation, supporting process options not accessible to conventional process technologies and ultimately resulting in enhanced process performance (1-3). Matching of the reactor fluids to the requirements of the particular reaction studied may also contribute to improved process control and thus product quality. The suggested use of microreactors has been analyzed and demonstrated at different scales of operation. There is now good evidence from different studies that microreactors offer high potential to be applied in the industrial production of fine chemicals and pharmaceuticals (1, 2). Biocatalysis has been receiving increased attention for process development in the chemical industries (4). A large number of catalytic transformations are nowadays carried out advantageously by using enzymes instead of purely chemical catalysts, especially in situations where selectivity is a key issue. Interest has therefore been high lately in integrating biocatalysis with microreaction technology (5-7). There are several potential advantages that result from use of microreactors for biocatalytic synthesis. These can be categorized broadly according to whether benefits are realized in the development phase or during the actual production. As already mentioned, one possibility is that the microreactor is used for screening purpose. Accurate selection of enzyme from an often large variety of candidates is a critical step of modern biocatalytic process development. Following an initial selection of candidates that is usually based on high-throughput screening and is carried out typically in microwell or even further miniaturized formats (e.g. microdroplets), intermediate selection of promising enzymes might be done using continuously operated microreactors (6, 8, 9). One particular advantage of using microreactors at this stage is that controlled conditions of continuous process operation can be applied. The high-throughput work usually screens for molecular properties of the enzyme, such as bulk stability or selectivity, but it is extremely important that enzyme selection is grounded on enzyme performance under process-near conditions. Experiments done in microreactors can provide the critical evidence. Another opportunity resulting from the use of microreactors in the development phase is quality-controlled supply of material (e.g. pharmaceutical ingredients) for product evaluation and clinical testing (7). The microreactor might constitute a flexible system with which to carry out biocatalytic synthesis on demand. However, in the production phase the use of microreactors must be justified by a clear cost advantage. Reduction in capital expenditure and reduction in operating costs are potential benefits resulting from the use of microreaction technology, and process intensification is the usual way to achieve them (7). Process intensification was defined as the dramatic decrease, typically by two magnitude orders or more, in physical size of a reactor while accomplishing a given production objective. It is generally thought to be best achievable through precise matching of reactor fluidics to the specific requirements of the transformation analyzed. The potential for such process intensification is not always clear in biocatalytic reactions (7, 10). Typical turnover frequencies ($k_{cat}$) for a variety of enzymatic conversions are in the range 1 – 100 s⁻¹. Using the
whether the steps of the immobilization can be done outside of the microreactor when beads are used, or they must be done in situ and therefore most often under flow when immobilization occurs directly on the reactor’s internal microstructure (6, 9, 11).

Secondly, the mode of attachment of the enzyme on the support needs to be defined, requiring selection from a large number of possibilities developed for enzyme immobilization in conventional reactors (12).

Biotransformations performed under conditions of continuous flow are usually built around immobilized enzymes, representing the most widely used form of heterogeneous biocatalyst applied at the industrial scale (4). Immobilization typically involves covalent or non-covalent stable attachment of the enzyme onto the surface of a solid carrier. Enzyme is therefore retained in the system and the total turnover number of the biocatalyst (mole product formed/mole enzyme used) is enhanced. Macroporous beads having an overall diameter of several hundreds of micrometer are often used as carriers, which are then applied in different reactor configurations (e.g. stirred tank, fixed or fluidized bed) (4). The defining feature of microreactors that their operation involves a continuous fluid phase implies that enzymes are preferably utilized in an immobilized state, and the immobilization therefore constitutes a key unit operation in the development of biocatalytic microreactors (7, 9, 11). One possibility is to adopt standard immobilization protocols that involve attachment of enzyme onto beads. Thus biocatalytically functionalized microbeads might be assembled in microchannels to generate an enzymatic micro-fixed bed (6, 11). Direct immobilization of enzyme on the surface of the internal microstructure of the microreactor (e.g. microchannel wall) is an alternative option (6, 11). Enzyme immobilization should be stable under conditions of fluid flow, but also reversible on demand, when immobilized inactive enzyme needs to be replaced by fresh enzyme, for example (7). It should also provide a high binding capacity so that a sufficient amount of enzyme can be attached on the internally available surface area. Ideally, the immobilization would be highly selective, thus allowing target enzyme to be captured from a complex protein mixture without the requirement for protein purification prior to immobilization (7). Under conditions where the microreactor is used for screening, there will not be enough time to isolate each enzyme candidate and therefore, selective immobilization will probably be a critical feature (7, 10, 11). Here we summarize recent advances in the field of enzyme immobilization in microreactors.

ENZYME IMMOBILIZATION IN MICROSTRUCTURED REACTORS

Development of any enzyme immobilization on solid support requires specification of two process parameters in particular. First of all and probably most importantly, the type and the form of the support need to be chosen, keeping in mind that in microreactors the decision determines the applicable type of reactor configuration (e.g. micro packed bed reactor, monolithic microreactor) (4). The decision also determines

inverse of $k_{cat}$, we calculate that the characteristic times of enzymatic reactions lie between 0.01 and 1 sec, implying that biotransformations in homogeneous solution will normally not be limited by mass transfer. However, many biotransformations involve transport across phase boundaries, as for example in heterogeneously catalyzed reactions where enzyme immobilized on solid support is applied or in liquid-liquid and gas-liquid two-phase reactions (7). Accelerating the mass transfer can therefore have significant effects on the overall transformation rate in these reactions, as shown in several studies. The use of microreactors might therefore contribute to process intensification in these cases (7).

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immobilization under flow should not present a problem. The wall shear rate may have to be considered. Wall shear could have negative effects on enzyme immobilization, especially in cases where the overall immobilization initially involves weak adsorption of the enzyme to the wall surface. Enhanced wall shear could result in unwanted release of the bound enzyme back to the liquid phase. It would be interesting therefore to evaluate immobilization procedures in microstructured elements under flow (using different flow rates) and compare their performance to an otherwise identical reference on micro beads. Additionally, it is important to keep in mind that “internal” immobilization limits one to working with the material applied for microreactor fabrication. Reduced pressure drop, lowered risk of clogging, and comparably well-defined flow pattern present clear advantages of microreactors having enzymes immobilized on the walls of their internal microstructure (10, 11). Enzyme immobilization on the internal surface could involve more or less monolayer binding of the protein to the surface or it could occur through coating the walls with a micron-thick proteinaceous layer (11). Layer deposition could involve direct polymerization of the protein or wash-coating procedures such as sol-gel immobilization (20). The operating flow conditions for coated wall microreactors resemble somewhat the conditions used in perfusion chromatography where the flow-through pores of the chromatographic material represent the microchannels in the microreactor, and the diffusive pores in chromatography have analogy to diffusive pores in the wall layer.

Methods of enzyme immobilization can be classified broadly according to whether they involve direct binding of the enzyme to the surface of the support or apply another mode of attachment to the surface (e.g. coating) (7, 11). Direct binding can be further categorized according to whether covalent bond formation is involved. Amino acid residues on the protein surface are mainly used for covalent attachment whereby lysine (amino group), arginine (guanidino group), aspartic acid (carboxylic acid group), and glutamic acid (carboxylic acid group) residues are prime targets for covalent coupling chemistry. Due to its high reactivity, cystein (sulphydryl group) is also interesting for covalent bond formation. However, cysteins are not normally found on the protein surface. Non-covalent adsorption to a surface usually involves hydrophobic and polar-charged interactions as its main attractive force. Bio-specific or bio-mimetic interactions are sometimes used to enhance the specificity of protein binding to the surface. Protein engineering is applied to raise specificity in binding, but also to improve the overall affinity and the binding capacity. With increasing specificity, protein adsorption to the surface is expected to gradually change from a rather random process that results in multiple orientations of the bound protein (“random immobilization”) to one where a single orientation is highly preferred (“oriented immobilization”) (7). An interesting approach of protein engineering is to append a specific “binding element” to the enzyme of interest. This binding element, which can be a short peptide of typically 10 – 15 amino acids or a discrete protein domain, is fused to the N- or C-terminal end of the enzyme. The resulting so-called chimeric protein now contains two functionalities, one for enzymatic catalysis and another for binding to a particular surface (7). Well known binding
Table 1. Summary of methods for enzyme immobilization in microreactors.

<table>
<thead>
<tr>
<th>Material</th>
<th>Configuration</th>
<th>Immobilization technique</th>
<th>Surface modification</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silica micropillar</td>
<td>Particle</td>
<td>Enzyme immobilized into beads or monoliths packed into capillary</td>
<td>Surface activation by silanization; usually glutaraldehyde cross-linking; other possible functionalization</td>
<td>(7, 10, 11 and refs here)</td>
</tr>
<tr>
<td>Silica microcatalyst</td>
<td>Covalent</td>
<td>Covalent binding to the inner tube surface</td>
<td>Surface activation by silanization; usually glutaraldehyde cross-linking; other possible functionalization</td>
<td>(7, 10, 11 and refs here)</td>
</tr>
<tr>
<td>Silica microcatalyst</td>
<td>Surface</td>
<td>Immobilization on thin film of mesoporous silica deposited on walls of boronosilicate tubes</td>
<td>Physical adsorption; larger immobilization leading to other possible functionalization</td>
<td>(7, 10, 11 and refs here)</td>
</tr>
<tr>
<td>Silica micropillar</td>
<td>Cross-linking</td>
<td></td>
<td>Pd-silica (sol-gel modified)</td>
<td>(7, 10, 11 and refs here)</td>
</tr>
<tr>
<td>Particle entrainment</td>
<td>Enzyme</td>
<td>Enzyme immobilized into monolith or glass particles packed in microchannels, covalent or reversible adsorption</td>
<td>Surface activation by silanization; usually glutaraldehyde cross-linking; other possible functionalization</td>
<td>(7, 10, 11 and refs here)</td>
</tr>
<tr>
<td>Micromass plate</td>
<td>Covalent</td>
<td>Covalent binding on surface</td>
<td>Surface activation by silanization; usually glutaraldehyde cross-linking; other possible functionalization</td>
<td>(10, 11, 12 and refs here)</td>
</tr>
<tr>
<td>Immobilization of microchannel</td>
<td>Physical</td>
<td>Physical adsorption</td>
<td>Multi-bin conjugation; other possible functionalization</td>
<td>(9, 10, 11 and refs here)</td>
</tr>
<tr>
<td>Immobilization of microchannel</td>
<td>Immobilization on membrane, using adsorption or covalent binding</td>
<td>Larisieratable immobilization leading to other possible functionalization</td>
<td>(15)</td>
<td></td>
</tr>
<tr>
<td>Immobilization of microchannel</td>
<td>Covalent</td>
<td>Covalent immobilization on polymer brushes coating the surface</td>
<td>Pd-silica (sol-gel modified); covalent attachment or His-tag binding</td>
<td>(11 and refs here)</td>
</tr>
<tr>
<td>Glass monolithic composite</td>
<td>Complex</td>
<td>Complex formation; Ni-NTA surface and protein His-tag</td>
<td>Purification and immobilization method; other possible functionalization</td>
<td>(19)</td>
</tr>
<tr>
<td>Rascher plate</td>
<td>Covalent</td>
<td>Covalent binding</td>
<td>Glutaraldehyde cross-linking; other possible functionalization</td>
<td>(19)</td>
</tr>
<tr>
<td>Rascher plate</td>
<td>Covalent</td>
<td>Covalent binding</td>
<td>Glutaraldehyde cross-linking; other possible functionalization</td>
<td>(19)</td>
</tr>
<tr>
<td>Metal-based</td>
<td>Covalent</td>
<td>Covalent binding on silanized aluminum oxide surface</td>
<td>Larger surface for immobilization; glutaraldehyde cross-linking; other possible functionalization</td>
<td>(23, 24)</td>
</tr>
<tr>
<td>Metal-based</td>
<td>Covalent</td>
<td>Covalent binding on silanized aluminum oxide surface</td>
<td>Larger surface for immobilization; glutaraldehyde cross-linking; other possible functionalization</td>
<td>(23, 24)</td>
</tr>
<tr>
<td>Organic polymer-based</td>
<td>Complex</td>
<td>Ni-NTA surface and protein His-tag Purification and immobilization method; other possible functionalization</td>
<td>Ni-NTA surface and protein His-tag Purification and immobilization method; other possible functionalization</td>
<td>(36)</td>
</tr>
<tr>
<td>Organic polymer-based</td>
<td>Particle</td>
<td>Enzyme immobilized in silica nanoparticle: polymer, ceramic or silica monolith in situ formed, polylysine beads, magnetic beads or agarose beads</td>
<td>Glutaraldehyde cross-linking; other possible functionalization</td>
<td>(15, 16, 17)</td>
</tr>
<tr>
<td>Organic polymer-based</td>
<td>Covalent</td>
<td>Covalent binding on surface</td>
<td>Glutaraldehyde cross-linking; other possible functionalization</td>
<td>(15, 16, 17)</td>
</tr>
<tr>
<td>Organic polymer-based</td>
<td>Immobilization on a membrane, using adsorption or covalent binding</td>
<td>Glutaraldehyde cross-linking; other possible functionalization</td>
<td>(15, 16, 17)</td>
<td></td>
</tr>
<tr>
<td>Ceramic-based</td>
<td>Reversible</td>
<td>Reversible immobilization on surface activated monolith</td>
<td>Surface activation by silanization; usually glutaraldehyde cross-linking; other possible functionalization</td>
<td>(7 and refs here)</td>
</tr>
</tbody>
</table>

The table is not meant to be comprehensive, but it is rather used to give a representative overview.
Surface coating for enzyme immobilization presents an interesting development in field of biocatalytic microreactors. A particular advantage of surface coating is that the specific catalytic surface is increased in comparison to the uncoated surface, so that the total amount of catalyst deposited on the microstructured surface can be enhanced substantially (21-24). A relatively simple approach is to deposit cross-linked enzyme aggregates on the surface (7, 11, 20). However, it can be difficult to control the aggregation such that a homogeneous and mechanically stable protein layer is formed on the surface. Moreover, enzyme activity may be lost to a large extent under the aggregation conditions. A general problem in polymerization or aggregation-based surface coating is that flow channels of the microstructure become clogged by precipitated material (7). Polymer brushes present an interesting alternative (25). A polymer brush is a layer of polymers attached with one end to a surface. Polymer brushes have been widely applied for anchoring enzymes and proteins onto flat gold or silicon surfaces (26, 27). Poly(methacrylic acid) (PMAA) polymer brush layer was formed on the interior of silicon-glass microreactor, followed by surface modification and lipase immobilization in under flow conditions (28). Technologies for the generation of patterned mesoporous silica thin films, silicon dioxide nanosprings, well-aligned 2D hexagonal mesoporous silica thin films, or porous aluminium oxide layers within microchannels have been developed and used for the immobilization of enzymes (14, 21-24, 29, 30).

The use of monolithic material has presented biocatalytic microreactor technologies with several new opportunities. Flexible use of reactor configurations is supported, and monoliths are also well compatible with enzyme immobilization (31-35). The modular Raschig ring reactor is an interesting development in the field (36). Monoliths are incorporated into Raschig rings, which in turn are aligned on a perforated tube. The resulting reactor is flexibly adaptable for scale by varying the number of rings, but also for different chemistries by assembling rings providing different catalytic functionalities. Monolithic polymer displaying Ni-NTA on the surface was applied for automated purification and reversible immobilization of His-tagged proteins. Several examples of chemical syntheses using biocatalytic Raschig ring reactors have been published (7, 36). A similar approach could in principle be extended to multistep enzyme-catalyzed transformations, using one Raschig ring containing immobilized enzyme for each biocatalytic step (7). Recently, monolithic silica rods exhibiting very open and uniform 3D hierarchical pore structure of flow-through macropores and 20 nm mesopores have been synthesized. This material was applied for fabrication of a multichannel flow microreactor (37). Invertase was covalently attached using glutaraldehyde cross-linking to the microstructure, and enzymatic hydrolysis of sucrose was examined under flow conditions. Silica microstructured fiber providing a large surface area for covalent immobilization of Candida antartica lipase has been used for lipids transformations (38). It follows from the considerations above that a useful method of enzyme immobilization in microreactors would involve reversibility and specificity in protein binding to the surface. Oriented binding of the protein in good operational stability would be another important feature. Biological affinity could be employed to achieve reversible immobilization, and the bioin-avadin system is a good example (11, 39, 40). Biotinylated polylysine was physically attached on a glass surface to promote non-covalent, hence reversible immobilization of streptavidin-conjugated alkaline phosphatase. A microreactor developed from this immobilization was used for rapid determination of enzyme kinetics. Biotinylated lipid bilayer and partial biotinylation by photo patterning on fibrinogen were also used for immobilization. The oligohistidine tag in proteins is well known to bind to immobilized metal ions (Cu²⁺, Ni²⁺, Zn²⁺) that are presented on the surface by a chelating agent such as nitrilotriacetic acid. Various materials can be modified to display nitrilotriacetic acid surface groups, and several authors have already exploited affinity for surface-bound metals for immobilization of His-tagged enzymes in microreactors (7, 11, 41). However, the method still needs to be evaluated more carefully with respect to selectivity and strength of binding in dependence on the degree of surface activation. The catalytic effectiveness of the immobilized enzyme and the ease of re-use of microstructured elements need to be analyzed. Oligoarginine tags present another, currently not well explored opportunity for reversible immobilization. They might be useful, especially if direct immobilization on glass is considered (7). Surface binding modules differ from oligopeptide tags in that they are by themselves stable protein entities. Fusion proteins composed of target enzyme and surface binding module are therefore interesting candidates for immobilization in microreactors. The complexity of the immobilization procedure also needs to be considered, ideally, underivatized material can be used, and reversible enzyme immobilization is done through simple “wash on” and “wash off” procedures (42, 43). Fusion proteins containing a specific surface binding module (or tag) hold great promise for development of microreactor platform technologies. Surface coating and layer-by-layer technologies are also powerful approaches. Supramolecular assembly presents another possibility. Reversible enzyme immobilization is a highly active field of research in biocatalytic microreactors (39, 44, 45).

REFERENCES AND NOTES

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