Assessment of intraparticle biocatalytic distributions as a tool in rational formulation

Jeroen van Roon*, Rik Beeftink†, Karin Schroën‡ and Hans Tramper§

Research has shown that the intraparticle biocatalytic distribution has extensive effects on the properties of various (industrial) biocatalytic particles and their performance in (bio-) chemical reactions. In recent years, advances in molecular chemistry have led to the development of many different specific (immuno-) labeling and light-microscopic detection techniques. Furthermore, high-quality image-digitizing devices and enhanced computing power have made image analysis readily accessible. These technologies may lead to the assessment and improvement of the internal biocatalyst profile as an integral part of biocatalytic particle optimization.

Addresses
Wageningen University, Department of Agrotechnology and Food Sciences, Food and Bioprocess Engineering Group, PO Box 8129, 6700 EV Wageningen, The Netherlands
*e-mail: jeroen.vanroon@algemeen.pk.wau.nl
†e-mail: rik.beeftink@algemeen.pk.wau.nl
‡e-mail: karin.schroen@algemeen.pk.wau.nl
§e-mail: hans.tramper@algemeen.pk.wau.nl

Current Opinion in Biotechnology 2002, **13**:398–405

0958-1669/02/$ – see front matter

Published online 25 June 2002

Abbreviations

CLEC	cross-linked enzyme crystal
CLSM	confocal laser scanning microscopy
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
SEM	scanning electron microscopy
TEM	transmission electron microscopy

Introduction

Biocatalysts have been shown to be of great industrial importance over the past decade. In the literature, many new biocatalysts and their applications can be found and their number is still expanding rapidly. The development of new biocatalyst particles partly remains a craft: usually many alternative formulations are made and the best for the specific application is selected. Recently, however, more attention has been given to the rational tailor-made design of industrial biocatalytic particles. An important design parameter is the distribution of the biocatalyst within the particle: the precise position of the catalytic enzymes or cells. Knowledge obtained in histochemistry can be applied to this distribution. Here, various detection and labeling methods were developed to characterize the structure and heterogeneity of different structures, as well as the locus of certain compounds, organelles, cell types or (cell) structures within cells and tissues of plants, molds, animals and microorganisms [1–6]. In this review, an overview of various labeling and detection methods and their feasibility is given, as these techniques form the basis for the determination of the intraparticle activity distribution within immobilized biocatalysts. These distributions profoundly affect the macroscopic performance of biocatalytic particles.

Particles

Tramper *et al.* [7•] recently introduced a generic decision strategy to facilitate the choice of 'when and how to use biocatalysts'. Immobilization of the costly biocatalytic enzymes or cells facilitates reuse, possibly with extremely high enzyme or cell loadings, leading to high volumetric activities. Immobilization also creates the possibility to use the biocatalyst in a packed-bed or fluidized-bed reactor [8].

The properties of the immobilized biocatalysts depend on the properties of both the enzyme or cell and the support material [9•]. The combination of both can yield biocatalysts with a 100-fold increase or decrease in the activity [10]. Inside the biocatalytic particle mass transfer of substrates and products can become limiting, because cells or enzymes are packed tightly into small volumes (or onto small surfaces). Diffusional limitation causes gradients of substrates and or products in the catalyst particle.

Immobilization can lead to various positive effects that are observed in the particle surrounding media. An immobilized biocatalyst can (apparently) be very stable. Many papers report improved operational stability upon immobilization [10–16]. Similarly, increased thermal stability upon immobilization has also been reported [8,14,15].

Unfortunately, diffusional limitations also cause negative side-effects. The effectivity of a biocatalytic particle with diffusion limitation, in general, is lower than that of the native biocatalyst. Diffusional limitation can also alter the (apparent) substrate specificity [11,15]. In the case of multisubstrate conversions, additional disadvantages can be mentioned. Litjens *et al.* [17] showed that diffusional limitation causes decreased enantioselectivity and Schroën *et al.* [18] found that during the production of the semisynthetic antibiotic cephalexin a higher enzyme loading results in lowered specificity and effectivity of the reaction.

Intraparticle distributions

Often, the biocatalyst is assumed to be homogeneously distributed over the particle. Sometimes efforts are actually made to accomplish this [19,20], whereas in other cases a homogenous distribution is simply inferred from the extended contact time during immobilization. Experimental confirmation of this assumption, however, is omitted in nearly all cases. In other cases, the unknown active biocatalyst

profiles are estimated from observed kinetic macroscopic data in a fitting procedure [21]. Do and Hossain [22,23] applied the latter procedure by quasi-steady-state and deactivation analysis.

Scharer *et al.* [24] discussed an increased selectivity and effectivity for pellicular biocatalytic particles, at the cost of diminished stability as compared with homogeneously distributed biocatalysts. A quantitative description of the intraparticle distribution of catalytic activity might further substantiate their rationale. Polakovic *et al.* [20] determined the effective sucrose diffusivity in supposedly homogeneous biocatalytic particles by measuring the macroscopic product formation rate. Apart from the question as to whether this assumption is valid, this method cannot be applied to many industrial biocatalytic particles, because they possess an unknown, possibly heterogeneous, distribution. To determine effective diffusivity in this way, knowledge of enzyme distribution is essential.

Characterization of biocatalytic particles

So far, we have seen that the immobilization of a biocatalyst can be the cause of a variety of positive and negative effects. Recent research has focused on the specific use of a certain distribution to create a biocatalytic particle that meets the most important design criteria, while minimizing the negative consequences of immobilization.

Therefore, we will discuss recent advances in research on the structure and composition of biocatalytic particles. In particular, we focus on the determination of the intraparticle distribution of biocatalytic activity and various labeling and detection methods used to establish this. Important parameters are the desired specificity of labeling and the required resolution of detection.

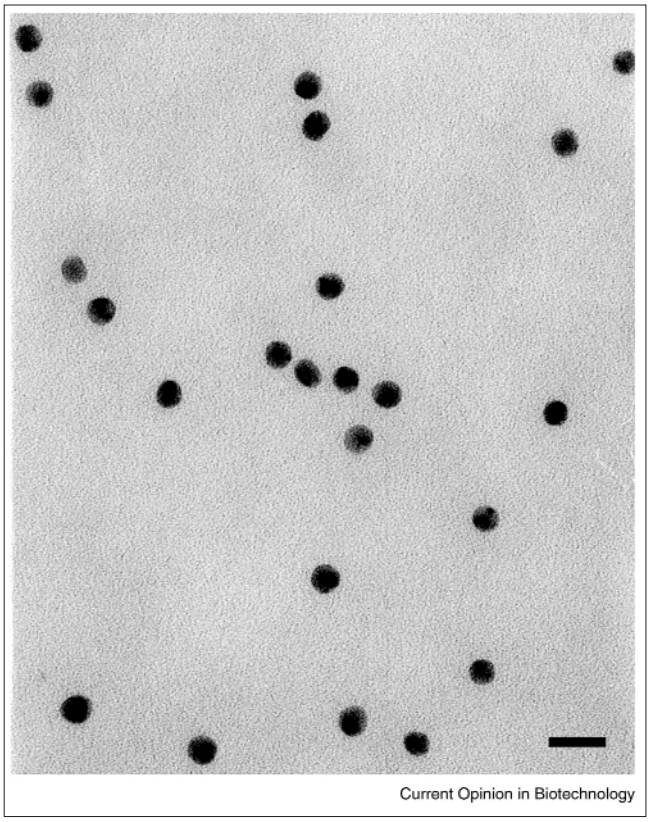
In general, assessment of the distribution consists of a few consecutive steps. Usually, the first step is the fixation and preparation of the material; if necessary, the material is embedded to facilitate subsequent sectioning. In the second step, the particle is broken or cut into sections to access its interior (non-invasive methods do not require this step). A labeling step is then performed. In this step in particular, resolution is an important issue. Several questions need to be addressed: what kind of chemical is to be labeled, how specifically does it need to be labeled, and what resolution is required? Since the choices of labeling and detection methods are interdependent, they are discussed simultaneously. After labeling and final observation, image analysis can be performed: images are subjected to different mathematical algorithms in order to quantify distributions of labeled species.

Preparation, fixation and embedding

Chemical fixation and embedding

Fixation of the biocatalyst can be performed to minimize (ultra)structural damage from subsequent procedures. In chemical fixation, proteins can be covalently cross-linked by using classical fixates like glutaric dialdehyde and formaldehyde. A general problem with chemical fixation is the potential loss of epitopes (antibody-binding sites) for specific labeling. Subsequently, the fixated structures can be embedded to enable the biocatalytic particle to be cut into sections. Usually, one of the first steps in embedding is dewatering of the sample in a series of solvent concentrations.

Figure 1



TEM photograph of an ultrathin immunogold-labeled cross-section of immobilized penicillin G acylase, unpublished work. Bar corresponds to 20 nm.

After dehydration, a resin can be added. Acrylic resins are usually the most suited for subsequent immunolabeling, as epoxy resins can bind covalently to biological materials, particularly with proteins [25]. Epoxy resins are known to give better preservation of ultrastructural details than acrylic sections, because they are more stable when exposed to an electric beam and are easier to cut. Brorson and colleagues [26,27] describe some recent methods to improve immunolabeling with epoxy resins.

Physical fixation and embedding

Physical particle fixation can be done by freezing. An excellent structure-conserving fixation technique is high-pressure freezing (200 bar) [28,29]; however, this treatment is not particularly well suited for epitope preservation. In cases where subsequent labeling is required, the sample can be frozen rapidly at ambient pressures by plunge-freezing (e.g. in liquid nitrogen, ethane or propane). This cryofixation also

Figure 2

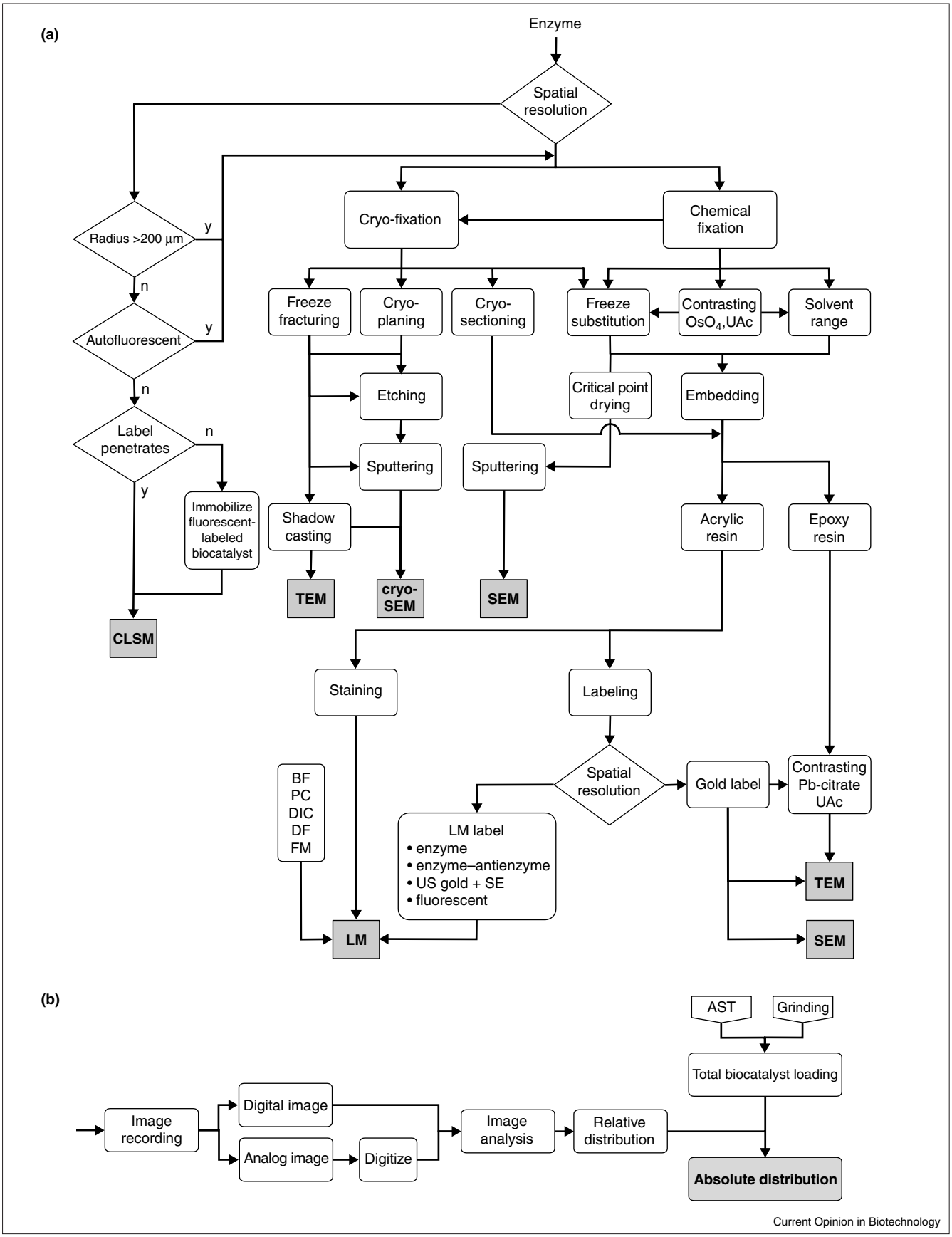

(a)
Enzyme
Spatial resolution
Radius >200 μm
y
n
Autofluorescent
y
n
Label penetrates
n
y
Immobilize fluorescent-labeled biocatalyst
CLSM
Cryo-fixation
Chemical fixation
Freeze fracturing
Cryo-planing
Cryo-sectioning
Freeze substitution
Contrasting OsO_4,UAc
Solvent range
Etching
Critical point drying
Embedding
Sputtering
Sputtering
Shadow casting
Acrylic resin
Epoxy resin
TEM
cryo-SEM
SEM
Staining
Labeling
BF
PC
DIC
DF
FM
Spatial resolution
Gold label
Contrasting Pb-citrate UAc
LM label
• enzyme
• enzyme–antienzyme
• US gold + SE
• fluorescent
TEM
SEM
LM
(b)
AST
Grinding
Total biocatalyst loading
Image recording
Digital image
Analog image
Digitize
Image analysis
Relative distribution
Absolute distribution
Current Opinion in Biotechnology

Figure 2 legend

Decision schemes for choosing a suitable localization technique: **(a)** detection (see text) and **(b)** image processing (see text). AST, active-site titration; BF, bright-field microscopy; DIC, differential interference contrast microscopy; DF, dark-field microscopy; FM, fluorescence microscopy; LM, light microscopy; PC, phase-contrast microscopy; SE, silver enhancement.

serves as an embedding step in which the frozen water replaces the resin. Subsequently, the frozen material can be cut into sections by cryo-sectioning (e.g. [9•,30]) to study the inner structures with a cryo-microtome or an ultracryo-microtome. A recently developed technique is cryo-planing by ultracryo-microtomy [31•], in which the frozen sample is cut with a diamond knife. The sliced sections themselves are ignored, whereas the exposed sample surface is extremely flat and therefore very suitable for use in (cryo)-scanning electron microscopy (SEM) studies. This is not always possible with older techniques like freeze-breaking, as the plane of fraction can be very irregular and could reveal little of the inner structures [32].

Hybrid fixation/embedding

A hybrid technique for fixation and embedding is freeze substitution [33]. In this technique, the material is frozen and the (amorphous) ice then replaced by a solvent, like iso-octane or heptane, by application of a specific temperature profile. This has proven to be a reliable technique, giving excellent preservation of the ultrastructure of the sample compared with other dehydrating and fixation methods. Another advantage is that the water-soluble components (sugars, amino acids, enzymes, etc) remain inside the sample.

Labeling

Now that the fixation and preparation of the sample is complete, a labeling step can be performed.

Groups of chemically related compounds can be stained. Stains are very efficient, cheap and easy to use. Proteins, for instance, can be stained with reagents like Coomassie brilliant blue or naphthol blue black [9], fats can be stained with phenol red, and phospholipids (in cell membranes) can be visualized with osmium tetraoxide or uranyl acetate. There are many more reagents for different kinds of chemical substances. Staining methods, however, are not very specific or selective. In the case of immobilized enzymes, for instance, staining cannot be used if the carrier material itself contains proteins. Furthermore, the enzyme solution to be immobilized often contains impurities that will be stained as well, and active enzymes cannot be distinguished from denatured, aggregated or precipitated enzymes.

Better selectivity can come from the use of chromogenic reference substrates, which can be used to determine the presence of enzymes that can utilize the chemical as a substrate (in contrast with the labeling of all proteins at once) [34].

During the past 10 years, developments in the technique of producing (monoclonal) antibodies and the (commercial) availability thereof, has led to an increase in the number of publications on histochemistry [35]. Antibodies are very specific and therefore used to detect very specific, well-defined molecules. They can be raised in, for example, rabbits, mice, rats and goats. Chickens are being increasingly used for antibody production, as their eggs can contain very high titers of antibody in the egg yolk (IgYs) [36], which makes bleeding of the animal obsolete. If primary labeling is used, the antibody also contains a specific label to make it visible. In secondary labeling, a secondary antibody, raised against the animal in which the first antibody was produced, contains the label for detection. Advantages of primary labeling are the relatively high labeling efficiency and the high target specificity compared with secondary labeling. Secondary labeling, however, is more flexible, as secondary antibodies raised against all antibodies of a certain animal are readily available with all kinds of labels.

The label attached to the antibody can be an enzyme that performs a specific reaction, from which it can be recognized upon addition of substrate for that enzyme. Alkaline phosphatase is used in many different fields of research, where enzyme distributions in different tissues are established [1,37,38]. It can hydrolyze naphthol phosphate esters like nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) to form phenolic compounds and phosphates. The phenols couple to colorless diazonium salts to produce insoluble azo dyes. The resolution of this procedure is limited, as multiple layers of the precipitated dyes are indistinguishable from monolayers. The coloring reaction can be studied by conventional light microscopy.

Also, enzyme antienzyme soluble complexes have been routinely employed in histochemical techniques [39,40]. The soluble complex of peroxidase antiperoxidase (PAP) consists of three peroxidases and two antiperoxidase subunits in a cyclic structure. The complex can oxidize a substrate, which produces a brown end product that is highly insoluble in ethanol and other organic solvents.

An ultrasensitive chemoluminescent imaging system (capable of detecting single photons) for quantitative analysis and visualization of the spatial distribution of biomolecules (such as enzymes) in tissues and cells was presented by Roda *et al.* [41].

Another commonly employed label is gold, which is often used in combination with electron microscopy. When extremely high resolutions are required, detection at the molecular level (e.g. single enzymes) is possible

by immunogold labeling and subsequent analysis with transmission electron microscopy (TEM) [42]. This requires ultrathin sections of approximately 50–100 nm to yield an electron transparent coupe. Figure 1 shows a TEM photograph of an ultrathin immunogold-labeled cross-section of immobilized penicillin G acylase.

Immunogold-labeled sections can alternatively be studied by SEM. With this technique, structural information from topographic detection can be combined with the localization of enzymes by backscatter detection of a single sample. Immunogold labeling can also be used in conjunction with light microscopic techniques [43]. Because the gold particles (usually 10 nm and smaller) are far too small to be detected in the light microscope, a silver-enhancement step is often used. During silver-enhancement, metallic silver is deposited on to ultrasmall gold particles of 1 nm.

For industrial biocatalysts, which are usually in the range of 0.1 mm to several millimeters in diameter, light microscopy with an optical resolution down to 200 nm can already yield high detail. Combined with a highly specific labeling technique, such as immunogold or alkaline phosphatase labeling, this provides a powerful tool for the quantitative localization of enzymes in industrial biocatalysts: large areas can be viewed in one single image with light microscopy with relatively cheap and easily accessible equipment. Improvements in digital imaging and analysis software and the availability of increased computing power have led to a shift from classical light microscopic techniques (bright-field, phase-contrast, differential interference, dark-ground and polarization) towards fluorescence and confocal laser scanning microscopy (CLSM).

The fluorescence microscope usually contains at least two light sources: one provides normal illumination and the other excites the fluorochromes, most commonly by epi-illumination. Components can fluoresce in natural form (primary or autofluorescence) or after treatment with fluorescent dyes.

Fluorescent labels are relatively photostable, photosensitive and may be highly specific when linked to various macromolecules [44]. Among many others, fluorescein isothiocyanate (FITC) is a very well known green-emitting fluorochrome. When excited, however, it produces free radicals, which can be very harmful to the biocatalyst. Furthermore, FITC is pH-sensitive and bleaches very quickly. More stable and brighter dyes are, for example, Alexa 488 or Cy2. Also, red probes such as Alexa 568, Cy3, Rhodamine or Texas Red are used frequently. Green fluorescent protein (GFP) is a 27 kDa protein from the jellyfish *Aequorea victoria*, which fluoresces in the green part of the spectrum upon illumination with UV light. GFP is a useful tool for labeling cellular proteins to follow their spatial and temporal localization in living cells under a variety of experimental conditions [45]. Many other specific fluorescent labels or combinations of labels can also be used; an example is a hybrid label containing a fluorescent label and a gold label for simultaneous fluorescence microscopy and electron microscopy.

CLSM is a very important extension of fluorescence microscopy, because it is a non-invasive technique. As opposed to regular fluorescence microscopy, it analyses the interior instead of the surface of a sample. In confocal microscopy, a laser is used as a light source and the light is focused in a focal plane. The plane can be shifted over the depth axis to scan the sample optically. The advantage is clear: the sample does not need to be cut in sections and this avoids the risk of introducing artifacts by embedding and/or cutting [46].

The fluorescent labels can be introduced into the biocatalytic particles by labeling a biocatalyst with a fluorescent group before immobilization. One needs to be convinced, however, that the labeling of the biocatalyst does not affect its distribution upon immobilization, and this is usually hard to prove/establish. Other disadvantages include the limited penetration depth of CLSM of approximately 100–200 μm, owing to scattering and absorption of emitted light from the fluorochrome, and fading (quenching and bleaching), which reduces the emission intensity. A third problem is autofluorescence of the immobilization support material, which can interfere with the fluorescence of the fluorochrome.

Autofluorescence may also be beneficial. Prior and colleagues [47] used this phenomenon as a tool to section the embedded structure optically before actual sectioning with glass knives. In this way, they determined exactly at what depth the structures of interest were localized, so that they would not miss the area of specific interest in the middle of a section. Spiess and Kasche [48] used a combination of the pH-dependent emission of FITC and the pH-independent emission of tetramethylrhodamine isothiocyanate (TRITC) to measure the pH inside the carrier of immobilized enzymes during enzyme catalysis.

Image analysis

The data obtained in the labeling experiments can be analyzed by taking (digital) images. Without further manipulations, the detection yields a qualitative distribution or localization of the biocatalyst within the particle. When single particles are visible, they can be counted to obtain a relative quantitative distribution. When intensity differences are quantified by imaging software to obtain a (continuous) quantitative relative distribution, the background should be illuminated homogeneously or a correction must be made for it. Irregularly shaped particles can complicate the determination of a biocatalyst profile within the particle.

Relative quantitative differences in signal intensity can be combined with the total amount of biocatalyst in the particle to yield an absolute quantitative biocatalyst distribution

over the particle. Methods to determine the absolute amount of biocatalyst include activity measurements of the ground biocatalytic particles [49,50] and active-site titration [49,51].

Decisions

Research in molecular chemistry has led to the development of many different labeling and detection techniques. The availability of these techniques has provided scientists with a greatly expanded toolbox. In Figure 2, a scheme presenting different possibilities for labeling and detection techniques is given. Although incomplete, this could be used as a tool for deciding which labeling and detection methods might be used for the localization of enzymes in biocatalytic particles on a case-by-case basis; a similar scheme also applies to living cells.

Application of knowledge: current state of biocatalyst development

In recent years, numerous applications of rationally designed biocatalysts have been reported. Much research has focused on the development of new supports and on improved or new immobilization methods. Without being complete, some examples are given below.

Jing *et al.* [12] produced a mesoporous support with a well-ordered structure. Similarly, nanoporous sol-gel glass particles with a highly ordered structure were used by Wang and coworkers [16] to increase biocatalyst stability in organic solvents.

Efforts to improve covalent immobilization onto epoxy supports were made by Mateo *et al.* [52] by promoting physical adsorption onto the support before covalent binding. Carvalho and Cabral [53•] reviewed micelles as promising reaction media for lipases. Tischer and Kasche [9•] recently reviewed immobilization in non-aqueous media, and discuss the advantages of the use of cross-linked enzyme crystals (CLECs). Cao and colleagues [54,55] discuss a procedure that involves the physical aggregation of enzymes, followed by chemical cross-linking to form so-called cross-linked enzyme aggregates (CLEAs). They found these aggregates to have high volumetric activities combined with high specificity, they also exhibited higher stability and activity in organic solvents than compared with the corresponding CLEC.

Recently, research started on the actual control and assessment of the distribution of the biocatalyst in and over particles during immobilization in order to maximize the performance of (industrial) biocatalysts. Particles with a non-uniform distribution were produced that showed higher effectivities than more uniformly distributed particles [56••]. Martin dos Santos and colleagues [57,58•] exploited the diffusional limitation of oxygen to combine aerobic outer-region reactions with anaerobic reactions in the center of the same biocatalytic particle by controlled immobilization of aerobic and anaerobic cells within the same biocatalytic particle (the particles were called 'magic beads'). Krastanov [59] co-immobilized two enzymes for continuous phenol removal from waste streams and showed that the activities of the two enzymes complement one another.

Keusgen *et al.* [13] studied the immobilization of enzymes on polytetrafluoroethylene (PTFE) surfaces. These authors described a procedure to immobilize enzymes under physiological conditions using a spacer molecule; the spacer allows enzyme multilayers to be achieved and the protein layers can be renewed. Mateo *et al.* [60•] reported a fast and relatively simple method to reuse the support material several times by reversible immobilization of industrially relevant enzymes. Mohy Eldin and coauthors [61] researched the possibility of altering the hydrophobic/hydrophilic nature of the immobilized support by crafting cheap nylon particles. Using graft polymers, enzymes might be more available for reaction than is the case with enzyme entrapped in a gel.

Conclusions

For several decades, much research was conducted on various biocatalytic particles with very different compositions and properties. The optimization of such biocatalytic particles mainly was a craft: many formulations were made by trial and error and the one with the best case-specific properties was selected. This work led to valuable insights into how the distribution of the biocatalyst over the carrier can influence its behavior during a reaction.

In recent years, advances in molecular chemistry and histochemistry have led to the development of many different labeling and detection techniques. Highly specific labeling with antibodies has become common practice and a label can be chosen freely. Combined with the availability of high-quality image digitizing devices (charge-coupled device [CCD] cameras and scanners) and enhanced computing power, light microscopic techniques have become a powerful and readily available tool for the assessment of the internal biocatalyst profile at reasonable efforts and prices. This may lead to the acceptance of the assessment of the internal biocatalyst profile as an integral part of biocatalytic particle optimization.

Acknowledgements

DSM (Geleen, The Netherlands) and the Ministry of Economic Affairs of the Netherlands are kindly acknowledged for their financial support. The authors want to thank AC van Aelst and H Kieft of the Wageningen Laboratory of Plant Cell Biology for valuable discussions.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Voronin V, Touraev A, Kieft H, van Lammeren AAM, Heberle BE, Wilson C: **Temporal and tissue-specific expression of the tobacco ntf4 MAP kinase.** *Plant Mol Biol* 2001, **45**:679-689.

2. Pan J, Suzuki T, Ueda K, Tanaka K, Okubo M, Pan J: **Distribution of microbial mass and fibrolytic enzyme activities in different size of feed particles from rumen contents of sheep.** *Anim Sci J*, **72**:209-217.

3. Sugibayashi K, Hayashi T, Morimoto Y: **Simultaneous transport and metabolism of ethyl nicotinate in hairless rat skin after its topical application: the effect of enzyme distribution in skin.** *J Control Release* 1999, **62**:201-208.

4. Kato Y, Ooi R, Asano Y: **Distribution of aldoxime dehydratase in microorganisms.** *Appl Environ Microbiol* 2000, **66**:2290-2296.

5. Shores EM, Hunter MG: **Immunohistochemical localization of steroidogenic enzymes and comparison with hormone production during follicle development in the pig.** *Reprod Fertil Dev* 1999, **11**:337-344.

6. Denyer K, Dunlap F, Thorbjornsen T, Keeling P, Smith AM: **The major form of ADP-glucose pyrophosphorylase in maize endosperm is extra-plastidial.** *Plant Physiol.* 1996, **112**:779-785.

7. • Tramper J, Beeftink HH, Janssen AEM, Ooijkaas LP, VanRoon JL, Strubel M, Schroën CGPH: **Biocatalytic production of semi-synthetic cephalosporins: process technology and integration.** In *Synthesis of Lactam Antibiotics: Chemistry, Biocatalysis and Process Integration*. Edited by Bruggink A. Dordrecht: Kluwer Academic Publishers; 2001:207-250.

The authors present a generic decision strategy to facilitate the question 'When and how to use biocatalysts?' Although outside the scope of this review, the questions answered are complementary.

8. Calsavara LP, De Moraes FF, Zanin GM: **Comparison of catalytic properties of free and immobilized cellobiase Novozym 188.** *Appl Biochem Biotechnol* 2001, **91-93**:615-626.

9. • Tischer W, Kasche V: **Immobilized enzymes: crystals or carriers?** *Trends Biotechnol* 1999, **17**:326-335.

Discussion on the advantages and disadvantages of immobilized enzymes over soluble enzymes. Cross-linked enzyme crystals are also discussed.

10. Novick SJ, Dordick JS: **Investigating the effects of polymer chemistry on activity of biocatalytic plastic materials.** *Biotechnol Bioeng* 2000, **68**:665-671.

11. Cloix JF, Wainer IW: **Development of an immobilized brain glutamine synthetase liquid chromatographic stationary phase for on-line biochemical studies.** *J Chromatogr A* 2001, **913**:133-140.

12. Jing H, Li XF, Evans DG, Duan X, Li CY: **A new support for the immobilization of penicillin acylase.** *J Mol Catal B Enzym* 2000, **11**:45-53.

13. Keusgen M, Glodek J, Milka P, Krest I: **Immobilization of enzymes on PTFE surfaces.** *Biotechnol Bioeng* 2001, **72**:530-540.

14. Ragnitz K, Syldatk C, Pietzsch M: **Optimization of the immobilization parameters and operational stability of immobilized hydantoinase and L-N-carbamoylase from *Arthrobacter aurescens* for the production of optically pure L-amino acids.** *Enzyme Microb Technol* 2001, **28**:713-720.

15. Vertesi A, Simon LM, Kiss I, Szajani B: **Preparation, characterization and application of immobilized carboxypeptidase A.** *Enzyme Microb Technol* 1999, **25**:73-79.

16. Wang P, Dai S, Waezsada SD, Tsao AY, Davison BH: **Enzyme stabilization by covalent binding in nanoporous sol-gel glass for nonaqueous biocatalysis.** *Biotechnol Bioeng* 2001, **74**:249-255.

17. Litjens MJJ, Le KQ, Straathof AJJ, Jongejan JA, Heijnen JJ: **Diffusion limitation causes decreased enantioselectivity of esterification of 2-butanol by immobilized *Candida antarctica* lipase B.** *Biocat Biotrans* 2001, **19**:1-19.

18. Schroën CG, Fretz CB, DeBruin VH, Berendsen W, Moody HM, Roos EC, VanRoon JL, Kroon PJ, Strubel M, Janssen AEM, Tramper J: **Modelling of the enzymatic kinetically controlled synthesis of cephalexin — influence of diffusion limitation.** *Biotechnol Bioeng*: in press.

19. Handrikova G, Stefuca V, Polakovic M, Bales V: **Determination of effective diffusion coefficient of substrate in gel particles with immobilized biocatalyst.** *Enzyme Microb Technol* 1996, **18**:581-584.

20. Polakovic M, Kudlacova G, Stefuca V, Bales V: **Determination of sucrose effective diffusivity and intrinsic rate constant of hydrolysis catalysed by Ca-alginate entrapped cells.** *Chem* 2001, **56**:459-466.

21. Carvalho CML, Aires-Barros MR, Cabral JMS: **A continuous membrane bioreactor for ester synthesis in organic media: I. Operational characterization and stability.** *Biotechnol Bioeng* 2001, **72**:127-135.

22. Hossain MDM, Do DD: **General theory of determining intraparticle active immobilized enzyme distribution and rate parameters.** *Biotechnol Bioeng* 1989, **33**:963-975.

23. Do DD, Hossain MDM: **A novel method of determination of the internal enzyme distribution within porous solid supports and the deactivation rate constant.** *Biotechnol Bioeng* 1986, **28**:486-493.

24. Scharer A, Hossain MDM, Do DD: **Determination of total and active immobilized enzyme distribution in porous solid supports.** *Biotechnol Bioeng* 1992, **39**:679-687.

25. Kellenberger E, Durrenberger M, Villiger W, Carleman E, Wurtz E: **The efficiency of immunolabel on Lowicryl sections compared to theoretical predictions.** *J Histochem Cytochem* 1987, **35**:959-969.

26. Brorson SH: **Antigen detection on resin sections and methods for improving the immunogold labeling by manipulating the resin.** *Histol Histopathol* 1998, **13**:275-281.

27. Brorson SH: **How to examine the antigen-damaging effect of sodium ethoxide on deplasticized epoxy sections.** *J Histochem Cytochem* 1997, **45**:143-146.

28. Wang Y, Chen Y, Lavin C, Gretz MR: **Extracellular matrix assembly in diatoms (*Bacillariophyceae*). IV. Ultrastructure of *Achnanthes longipes* and *Cymbella cistula* as revealed by high-pressure freezing/freeze substitution and cryo-field emission scanning electron microscopy.** *J Phycol* 2000, **36**:367-378.

29. Mims CW, Rodriguez LC, Richardson EA: **Ultrastructure of the host-parasite interaction in leaves of *Duchesnea indica* infected by the rust fungus *Frommeela mexicana* var. indicae as revealed by high pressure freezing.** *Can J Bot* 2001, **79**:49-57.

30. Oyaas J, Storro I, Lysberg M, Svendsen H, Levine DW: **Determination of effective diffusion coefficients and distribution constants in polysaccharide gels with non-steady-state measurements.** *Biotechnol Bioeng* 1995, **47**:501-507.

31. • Nijsse J, van Aelst AC: **Cryo-planing for cryo-scanning electron microscopy.** *Scanning* 1999, **21**:372-378.

A study that compares and contrasts various cryo-planing techniques for low-temperature scanning electron microscopy. Modifications that improve the planing results are presented.

32. Walther P, Muller M: **Biological ultrastructure as revealed by high resolution cryo-SEM of block faces after cryo-sectioning.** *J Microsc* 1999, **196**:279-287.

33. Thijssen MH, Van Went JL, van Aelst AC: **Heptane and isooctane as embedding fluids for high-pressure freezing of Petunia ovules followed by freeze-substitution.** *J Microsc* 1998, **192**:228-235.

34. Alkema WBL, Floris R, Janssen DB: **The use of chromogenic reference substrates for the kinetic analysis of penicillin acylases.** *Anal Biochem* 1999, **275**:47-53.

35. Stoward PJ, Nakae Y, Van Noorden CJF: **The everchanging advances in enzyme histochemistry, 1986-1996**. *Eur J Histochem* 1998, **42 Special Issue**:35-40.

36. Tu YY, Chen CC, Chang HM: **Isolation of immunoglobulin in yolk (IgY) and rabbit serum immunoglobulin G (IgG) specific against bovine lactoferrin by immunoaffinity chromatography.** *Food Res Int* 2001, **34**:783-789.

37. Cheng XF, Wittich PE, Kieft H, Angenent G, XuHan X, van Lammeren AAM: **Temporal and spatial expression of MADS box genes, FBP7 and FBP11, during initiation and early development of ovules in wild type and mutant *Petunia hybrida*.** *Plant Biol* 2000, **2**:693-701.

38. Lawrenson JG, Reid AR, Finn TM, Orte C, Allt G: **Cerebral and pial microvessels: differential expression of γ-glutamyl transpeptidase and alkaline phosphatase.** *Anat Embryol (Berl)* 1999, **199**:29-34.

39. Kohsaka T, Sasada H, Sato E, Bamba K, Hashizume K: **Ultrastructural properties and immunolocalization of relaxin in the cytoplasmic electron-dense granules of large luteal cells during pregnancy in the cow.** *J Reprod Devel* 2001, **47**:217-225.

40. Najimi M, Bennis M, Moyse E, Chigr F: **Distribution of delta sleep-inducing peptide in the newborn and infant human hypothalamus: an immunohistochemical study.** *Biol Res* 2001, **34**:31-42.

41. Roda A, Pasini P, Baraldini M, Musiani M, Gentilomi G, Robert C: **Chemiluminescent imaging of enzyme-labeled probes using an optical microscope-videocamera luminograph.** *Anal Biochem* 1998, **257**:53-62.

42. Lovelock C, Lucocq J: **Quantitative immunoelectron microscopy reveals α2,6-sialyltransferase is concentrated in the central cisternae of rat hepatocyte Golgi apparatus.** *Eur J Cell Biol* 1998, **76**:18-24.

43. Castiglione MR: **Immunogold localization of trans-zeatin riboside in embryo and endosperm during early fruit drop of *Malus domestica*.** *Biologia Plantarum* 1998, **41**:523-532.

44. Spear RN, Cullen D, Andrews JH: **Fluorescent labels, confocal microscopy, and quantitative image analysis in study of fungal biology.** *Methods Enzymol* 1999, **307**:607-623.

45. Margolin W: **Green fluorescent protein as a reporter for macromolecular localization in bacterial cells.** *Methods Orlando* 2000, **20**:62-72.

46. Bougourd S, Marrison J, Haselhoff J: **An aniline blue staining procedure for confocal microscopy and 3D imaging of normal and perturbed cellular phenotypes in mature *Arabidopsis* embryos.** *Plant J* 2000, **24**:543-550.

47. Prior DAM, Oparka KJ, Roberts IM: **En bloc optical sectioning of resin-embedded specimens using a confocal laser scanning microscope.** *J Microsc* 1999, **193**:20-27.

48. Spiess AC, Kasche V: **Direct measurement of pH profiles in immobilized enzyme carriers during kinetically controlled synthesis using CLSM.** *Biotechnol Prog* 2001, **17**:294-303.

49. Janssen MHA, Vanlangen LM, Pereira SRM, Vanrantwijk F, Sheldon RA: **Evaluation of the performance of immobilised penicillin G acylase using active-site titration.** *Biotechnol Bioeng*: in press.

50. VanderWielen LAM: **Transients in the heterogeneous enzymatic deacylation of penicillin G.** In *A Countercurrent Adsorptive Fluidised Bed Reactor for Heterogeneous Bioconversions.* PhD thesis. Delft, The Netherlands; 1997:8.1-8.3.

51. Rotticci D, Norin T, Hult K, Martinelle M: **An active-site titration method for lipases.** *BBA Mol Cell Biol Lipids* 2000, **1483**:132-140.

52. Mateo C, Fernandez LG, Abian O, Fernandez LR, Guisan JM: **Multifunctional epoxy supports: a new tool to improve the covalent immobilization of proteins. The promotion of physical adsorptions of proteins on the supports before their covalent linkage.** *Biomacromolecules* 2000, **1**:739-745.

53. Carvalho CML, Cabral JMS: **Reverse micelles as reaction media for**
• **lipases.** *Biochimie* 2000, **82**:1063-1085.
Extensive review on the prospect of using reverse-micelles as reaction media for lipases. The authors give some fundamental aspects of lipase micro-encapsulation and review the progress that has been made in reversed micellar systems.

54. Cao L, van Langen LM, Van Rantwijk F, Sheldon RA: **Cross-linked aggregates of penicillin acylase: robust catalysts for the synthesis of β-lactam antibiotics.** *J Mol Catal B Enzym* 2001, **11**:665-670.

55. Cao L, Van Rantwijk F, Sheldon RA: **Cross-linked enzyme aggregates: a simple and effective method for the immobilization of penicillin acylase.** *Org Lett* 2000, **2**:1361-1364.

56. Ladero M, Santos A, Garcia-Ochoa F: **Diffusion and chemical**
•• **reaction rates with nonuniform enzyme distribution: an experimental approach.** *Biotechnol Bioeng* 2001, **72**:458-467.
The authors prepared biocatalytic particles with a non-uniform distribution of FITC-labeled β-galactosidase and measured the internal distribution with CLSM. They discuss a biocatalytic particle with a heterogeneous enzyme distribution that has higher effectiveness compared to particles with homogeneously distributed enzyme for the same Thiele modulus.

57. Martin dos Santos VAP: **Production and characterization of double-layer beads for co-immobilization of microbial cells.** In *Towards the Integration of Oxidative and Reductive Activities: Application to Nitrogen Removal by Co-immobilized Micro-organisms*. PhD thesis. Wageningen, The Netherlands; 2001:109-129.

58. Martin dos Santos VAP, Bruijnse M, Tramper J, Wijffels RH, Dos S:
• **V: The magic-bead concept: an integrated approach to nitrogen removal with co-immobilized micro-organisms.** *Appl Microbiol Biotechnol* 1996, **45**:447-453.
Presentation of a biocatalytic particle in which different microbial populations were selectively immobilized to integrate oxidative and reductive properties in a single particle.

59. Krastanov A: **Removal of phenols from mixtures by co-immobilized laccase/tyrosinase and Polyclar adsorption.** *J Ind Microbiol Biotechnol* 2000, **24**:383-388.

60. Mateo C, Abian O, Fernandez-Lafuente R, Guisan JM: **Reversible**
• **enzyme immobilization via a very strong and nondistorting ionic adsorption on support-polyethylenimine composites.** *Biotechnol Bioeng* 2000, **68**:98-105.
Presentation of tailor-made anionic exchange resins for very strong and reversible enzyme immobilisation to reduce costs of support materials.

61. Mohy Eldin MS, Schroën CGPH, Janssen AEM, Mita DG, Tramper J: **Immobilization of penicillin G acylase onto chemically grafted nylon particles.** *J Mol Catal B Enzym* 2000, **10**:445-451.