1. Definitions, Historical Aspects, and Perspectives

An application-related definition for immobilized enzymes was given by Chibata in 1978 [1]: they are “enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities which can be used repeatedly and continuously”; this also holds in essence for immobilized cells.

The immobilization of biocatalysts or other bioactive components often means their transformation from a soluble to an insoluble state by attaching them to an insoluble carrier material or by encapsulating the catalysts within a polymer matrix of synthetic or natural origin. This may result in a significant change of the native catalyst’s properties. For an explanation of the observed modified reaction behavior, the laws of heterogeneous kinetics have to be applied [2,3].

Examples of immobilized biocatalysts (enzymes and whole cells) were known since about 100 years. One of the first papers in this field was a report by Nelson and Griffin [4] on the adsorption of yeast invertase onto charcoal; the catalyst remained active in this state and catalyzed the hydrolysis of sucrose. However, the great practical importance of these findings was not recognized until several decades later; pioneering research began with the work of Grubhofer and Schleith [5], Bernfeld and Wan [6], or Katchalski-Katzir et al. [7], to name but a few. Since then, the number of publications increased significantly and research in this area of enzyme technology has not lost any of its topicality until to date. Application fields are numerous and steadily increasing. Immobilization of biocatalysts is not only important for an economical application of biocatalysts in (organic) synthesis, but also for the construction of biosensors or use of immobilized biocatalysts in wastewater treatment [8–10].

Furthermore, site-selective immobilization is a key step for on-chip enzyme assays, a topic excellently reviewed by Wong et al. [11]. A combination of biocatalysis with nanotechnology led, for example, in the recent past, to the development of nanocarriers for therapeutic proteins; these nanoconjugates are able to cross the blood–brain barrier and enable the treatment of diseases of the central nervous system (CNS), such as lysosomal storage diseases associated with cognitive impairment or CNS tumors [12–14].

An application of biocatalysts in immobilized form has several advantages: they may be reused several times, reducing the costs for a biocatalyst, and thus, of the entire process. The procedure often results in enhanced (thermal) stability, and spoiling of the obtained products is avoided. Regardless, up to now, large-scale applications of immobilized biocatalysts are still rather rare, and an immobilization procedure characterized by a general applicability does not seem to exist; the opinion that each biocatalyst needs a tailor-made immobilization procedure is widespread. Nonetheless, there are some outstanding examples of industrial application. The first one was the immobilization of aminoacylase by use of diethylaminoethyl (DEAE)-Sephadex as a solid support for the stereoselective hydrolysis of N-acyl-D,L-amino acids, developed in 1969 by Chibata et al., Tanabe Seiyaku Co., Japan [15], and the same group succeeded in establishing a large-scale industrial process for the amino acid L-aspartate production from ammonium fumarate by entrapment of L-asparagine ammonia-lyase-containing Escherichia coli cells [16,17]. Actual examples of industrial
processes performed in presence of robust immobilized enzymes were discussed by Thum et al. [18]. For further recently published reviews, see, e.g., Homaei et al. [19], Liese and Hilterhaus [20], Sheldon and van Pelt [21], Zucca and Sunjust [22], Mohamad et al. [23], and Kim et al. [24], as well as Rehm et al. [25] and Bernal et al. [26], with the latter two dealing with new strategies to integrate enzyme immobilization and protein engineering.

2. This Special Issue

When I was asked to overtake the role of a Guest Editor for this Special Issue, although feeling honored, I first declined against the background of other obligations. Now looking back on these months of acquiring possible authors for this project, I do not regret the invested time. This is particular due to the wonderful and uncomplicated cooperation with the Assistant Editor Adela Liao and her competent team. Moreover, I owe particular thanks to all the authors who contributed their excellent papers to this Special Issue that is comprised of twenty-nine articles, among them six reviews, covering many important aspects of this topic together with a variety of new approaches.

The enzymes, the immobilization of which is described in the different articles, belong to the enzyme classes EC 1 (glucose oxidase, cyclohexanone monoxygenase, horseradish peroxidase, ketoreductase, glucose dehydrogenase, and laccase), EC 2 (hypoxanthine–guanine–xanthine phosphoribosyltransferase and aminotransferase), EC 3 (cellulase, β-amylase, various lipases, invertase, endo-β-N-acetylglucosaminidase, β-fructofuranosidase, and the protease ficin), and to EC 4 (hydroxynitril lyase). Immobilized whole cells include those of *Escherichia coli*, Gram-positive *Planococcus* sp. S5, *Rhodotorula mucilaginosa*, Gram-negative *Enterobacter aerogenes*, *Komagataeibacter xylinum* B-12429 cells, and baker’s yeast lysates.

In more detail, Bracco et al. [27] immobilized the hydroxynitrile lyase from *Prunus amygdalus* on Celite R-633 for catalyzing very successfully the conversions of benzaldehyde to R-mandelonitrile; the immobilized enzyme was recycled up to ten times. A paper supporting glycoproteomic investigations was presented by Cohen et al. [28]. These researchers immobilized endo-β-N-acetylglucosaminidase from the commensal *Bifidobacterium infantis* for the release of bioactive N-glycans which might be of use as prebiotics or anti-pathogenic factors. The immobilized enzyme also catalyzed the cleavage of neutral glycans from whey proteins. Musa et al. [29] reported a low-temperature ethyl hexanoate synthesis by means of an acyl transfer reaction with an antarctic *Pseudomonas* AMS8 Lipase as catalyst in toluene that worked optimally at 20 °C. The product is employed as a synthetic flavor ester in the food, beverage, and cosmetic and pharmaceutical industries. According to Miguez et al. [30], a recombinant β-fructofuranosidase from the yeast *Xanthophyllomyces dendrorhous* that was expressed in *Pichia pastoris* turned out to be well suited for the synthesis of various neo-fructooligosaccharides such as neokestose, 1-kestose, neonystose, and blastose in a batch reactor at 30 °C. The β-fructofuranosidase was immobilized in polyvinyl alcohol lenticular particles, and could be reused for at least seven cycles without loss of activity. The tetrameric hypoxanthine–guanine–xanthine phosphoribosyltransferase from *Thermus thermophilus* HB8 was immobilized by del Arco et al. [31] onto glutaraldehyde-activated MagReSyn® Amine magnetic iron-oxide porous microparticles (MTHGXPR) by different strategies for the one-pot, one-step production of dietary nucleotides. The best variant showed negligible loss of activity at 60 °C during 24 h, and a reusability of up to seven cycles. Graphene oxide was grafted by Gao and coworkers [32] with the hydrophobic spacer p-β-sulfuric acid ester, ethyl sulfone aniline, for the covalent immobilization of cellulase. After optimizing the reaction conditions, the whole procedure was finished already after 10 min with an immobilization yield and efficiency of above 90%. At 50 °C, the half-life of the cellulase immobilized by this new approach was six-fold higher than that of the free cellulase. The potential this method may have for the immobilization of other enzymes needs to be clarified. Another new immobilization method described by Kovalenko et al. [33] makes use of SiO₂ xerogel and nanocarbons-in-silica composites for an entrapment of baker’s yeast lysates. The authors tested theses carriers with invertase and lipase activity of the enzymatically active components. The invertase activity increased by a factor of about six in the presence of multi-walled
carbon nanotubes (CNTs) inside the xerogel, whereas the lipase activity tested via esterification of fatty acids with aliphatic alcohols increased by a factor of nearly two. The lipase-active composite CNTs-in-silica biocatalysts operated without loss of activity for more than one thousand hours.

With the aim of degrading the nonsteroidal anti-inflammatory drug naproxen, Dzionek et al. [8] immobilized the Gram-positive bacterium Planococcus sp. S5 onto a loofah sponge. This conjugate degraded naproxen efficiently for 55 days without significant damage and disintegration of the carrier, and in higher concentrations than free cells did. Barley β-amylase is, among others, important for maltose production. Araujo-Silva et al. [34] prepared immobilized β-amylase in the form of cross-linked enzyme aggregates using bovine serum albumin or soy protein isolate as feeder proteins to reduce diffusion problems and to successfully obtain maltose from converting the residual starch contained in cassava bagasse. A two-enzyme system developed by Petrovićová and colleagues [35], consisting of ketoreductase and glucose dehydrogenase immobilized inside of polyvinyl alcohol (PVA) gel particles, served to catalyze the asymmetric reduction of keto esters to optically active hydroxy esters. The catalyst could be used 18 times with minimal loss of activity and complete conversion of a model substrate. The described storage conditions enabled the retention of 80% activity after about 10 months. High-throughput screening performed by Liu et al. [36] revealed Rhodotorula mucilaginosa as a very active and selective whole-cell biocatalyst for the asymmetric reduction of various aromatic ketones to the desired chiral alcohols. For cell immobilization, agar, calcium alginate, PVA-alginate, and chitosan were employed as support matrices; pH tolerance and thermal stability were improved. Cells immobilized on agar retained 90% activity after 60 days of storage at 4 °C and could be reused six times without loss of activity. Biological hydrogen production by microbial cells is an actual research area. Nakatani and coworkers [37], for this purpose, immobilized Enterobacter aerogenes expressing the ataA gene encoding the adhesive protein of Acinetobacter sp. Tol 5 on polyurethane foam, thereby simplifying and accelerating the immobilization of whole-cell catalysts. The total H₂ production was 0.6 mol/mol glucose at a production rate in the flow reactor of 42 mL·h⁻¹·L⁻¹.

An application example of biocatalyst immobilization for wastewater treatment was provided by Samoylova et al. [9]. They prepared stable cross-linked enzyme aggregates (CLEAs) of the thermostable esterase, estUT1, of the bacterium Ureibacillus thermosphaericus, and used these CLEAs for removal of the organophosphate insecticide, malathion. High operational stability and activity of the esterase was achieved by its coexpression with an E. coli chaperone team. Siar et al. [38] investigated experimental parameters influencing activity and stability of a ficin extract immobilized onto glutaraldehyde-activated agarose beads. An optimal immobilization yield with 80% was achieved at pH 9 and maximum enzyme loading was about 70 mg/g. Using casein as a substrate, the activity of the biocatalyst was 30%. The obtained results suggested a complex inactivation mechanism of the immobilized enzyme as a function of reaction conditions. An actual topic is the biocatalytic production of biodiesel. An attractive procedure reported by Adnan and coworkers [39] relies on a one-step encapsulation method for synthesizing X-shaped zeolitic imidazolate frameworks (ZIF-8) and immobilizing Rhizomucor miehei lipase. The conjugate showed a 26-fold increase in activity with soybean oil as a substrate compared to the free enzyme, and a conversion yield of 95.6% under optimal conditions; 84.7% of the initial activity was preserved after 10 cycles. Dell Antonio Facchini et al. [40] isolated and purified two Fusarium verticillioides lipases through adsorption using octadecyl Sepabeads and octyl Sepharose resin which resulted in a three-fold increase in activity. The catalysts were investigated with respect to their ability to release S-enantiomers from substrates, and their transesterification capacity. The obtained results indicate industrial applicability of the immobilized lipases. A new strategy to mitigate the often unfavorable interaction of enzymes with the solid support is the genetic fusion of T4-lysozyme to the N-terminus of enzymes to be immobilized. Planchestainer and coworkers [41] demonstrated with different enzyme-fusion conjugates that the T4-lysozyme acts as an inert shield in covalent immobilization and leads to the retention of or even significant improvement in the rescued activity.
Bergman and colleagues [42] provided a method for temporally resolving the transients of vesicular neurotransmitter release and fluctuations of metabolites such as glucose, thereby improving understanding of the regulation of neuronal activity. For this purpose, they developed an amperometric biosensor for co-detection of dopamine and glucose. The device enabled visualization of fluctuations in glucose and dopamine concentrations at a millisecond time scale. Komagataeibacter xylinum B-12429 cells immobilized in polyvinyl-alcohol cryogel turned out to be well suited for the production of bacterial crystalline cellulose from various renewable biomass sources as reported by Stepanov and Efremenko [43]. The cellulose passed through the pores of the cryogel matrix into the medium resulting in a continuous production. The immobilized cells retained 100% metabolic activity for at least 10 working cycles. An inorganic/organic hybrid support TiO$_2$–lignin was employed by Zdarta et al. [44] for the immobilization of a cellulase from Aspergillus niger. As is to be seen from the fact that the half-life of the immobilized cellulase was five times that of the free enzyme and over 90% of its initial catalytic was retained after ten repeated cycles, the hybrid support contributed to a significant improvement of the enzyme’s thermal and chemical stability.

Allertz et al. [10] presented the first application of commercial macroporous melamine formaldehyde foam Basotect® for covalent and adsorptive enzyme immobilization after pretreatment of the carrier surface. The immobilization conditions for laccase from Trametes versicolor and the lipase from Thermomyces lanuginosus were optimized, and the immobilized laccase was shown to be able to degrade 80% of the micro-pollutant bisphenol A in contaminated water. A novel matrix for enzyme immobilization, polyelectrolyte complex beads prepared from a two-step reaction of oppositely charged polymers, was developed by Krajčovič et al. [45] for entrapping recombinant E. coli cells overexpressing cyclohexanone monoxygenase. The immobilizate exhibited high operational stability and reusability, e.g., in connection with the oxidation of rac-bicyclo[3.2.0]hept-2-en-6-one to the respective lactones. Liang et al. [46] demonstrated with glucose oxidase (GOx) and horseradish peroxidase as examples that in situ self-assembly of Zn/adenine hybrid nano coordination polymers provides a simple one-step immobilization method, resulting in highly active catalysts with excellent reusability. The immobilized GOx could also be used in connection with a glucose biosensor, detecting the substrate selectively down to 1.84 µM.

This Special Issue also contains six review articles. Chapman et al. [47] provided an overview of the industrial application of (immobilized) biocatalysts in areas such as pharmaceutical, food, and beverage industries, as well as the energy sector (biofuel production and natural gas conversion). The authors discuss how the next generation of immobilized biocatalysts will be coined by the overlap of technical expertise in enzyme immobilization, protein, and process engineering to improve industrial processes with respect to product yield, market profitability, and environmental friendliness. Immobilized redox enzymes are employed for sensing applications, as well as for energy conversion. In this connection, direct electron transfer by tunneling between enzymes and conductive surfaces is of high importance. This requires controlling enzyme orientation at planar electrodes. Hitaishi et al. [48] review all the factors influencing a proper orientation together with various analytical methods for characterization and quantification of the desired orientation. The review contributed by Yamaguchi and colleagues [49] deals with techniques for the preparation of cross-linked enzyme aggregates (CLEAs), including co-immobilization of different enzymes. CLEAs often combine the advantages of immobilized enzymes such as high catalytic activities, good storage, and operational stabilities, as well as good reusability. A variety of biotechnological application examples are given demonstrating their economic and environmental benefits. Metal–organic frameworks (MOFs) are porous hybrid materials consisting of metal ions or clusters and organic ligands, and are characterized by a vast structural and functional tunability. Wang et al. [50] review these MOFs as platforms for enzyme immobilization, and the different applications of these enzyme–MOF hybrid materials. The improvement of catalytic activity and robustness of MOF-encapsulated enzymes compared to their free counterparts is discussed in detail. Consolidated bioprocessing (CBP) combines cellulases production, enzymatic hydrolysis, and microbial fermentation into a single operation to convert lignocellulosic biomass to platform
chemicals such as ethanol. Tabañag and colleagues [51] reviewed the contribution of different yeast-surface-display techniques for the creation of correspondingly engineered strains. Finally, Zdarta et al. [52] discuss the pros and cons of a large variety of inorganic and organic, as well as hybrid and composite, materials including nano supports, used for the immobilization of biocatalysts. The review was written with the aim of providing an orientation for selecting appropriate support materials with tailored properties for the preparation of highly effective biocatalytic systems and their application in different processes.

In conclusion, the Special Issue “Immobilized Biocatalysts” should be of great interest for all those involved in the various aspects of this topic, which are discussed in the contributions and review articles. They introduce new immobilization procedures, as well as novel support materials and applications, thereby meeting the state of the art of both scientific and technical standards.

Conflicts of Interest: The author declare no conflict of interest.

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