Bachelor's thesis

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The feasibility of nanomaterials for immobilizing enzymes utilized in biocatalysis

Bachelor's thesis in Chemistry
Supervisor: Odd Reidar Gautun
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Abstract

Enzymes have been used to catalyse chemical reactions for some time. Even though enzymes have proved to be very effective for some synthesises, the use of enzymes in synthetic chemistry have been limited due to limitations inherent to enzymes. The recent emerge of new nanomaterials and nanoparticles has reawakened the interest for chemoenzyatic synthesis. This thesis looks at the use of nanomaterials and nanoparticles to immobilize enzymes. Enzymes and relevant nanomaterials and nanoparticles will be reviewed as well as some immobilizing methods. The focus is shifted towards sustainability, "greenness", and industrial applicability. Nanobiocatalysis is found to have a promising potential. Nano-immobilized enzymes often show retained or improved kinetics, improved ease of separation from reaction mixture, and durability and reusability. This makes them superior to free enzymes. The most apparent disadvantage of nano-immobilized enzymes is their cost and therefore there is still modest use of nanobiocatalysis on a industrial scale. However, there is expected a reduction in nanomaterial production cost and consequently an increase in industrial applications in the future.

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1 Introduction

Recent development in biotechnology as well as nanotechnology and synthesis of nanostructures have given rise to the field of nanobiocatalysis.

Enzymes is highly relevant for use in synthetic chemistry due to their great properties regarding specificity, sustainability, and regio- and enantioselectivity.[1–3] This is an emerging field that shows great potential for enantiospecific drug synthesis, resolution of racemic mixtures etc. The large scale industrial use of enzymes is however somewhat limited due to the lack of stability of enzymes and their short lifetime.[4] Enzymes for use in the laboratory should be resistant to elevated temperatures, have a practical pH-range, have good solvent tolerance, and have low product inhibition.

By immobilizing enzymes onto a support matrix, it is possible to achieve improved properties such as chemical stability, resistance to higher temperatures, more extreme pH, and solvent tolerance.[3,4]

Nanomaterials and nanoparticles have emerged as a very interesting medium for enzyme immobilization. Many studies have found indications that enzymes immobilized on nanomaterials (and nanoparticles) have improved many of said properties.[5] Especially increased stability is highlighted. Increased chemical stability allow immobilized enzymes to be reused several times as well as allowing storage for a longer period of time. This is an important factor in the process of commercializing enzymes – making them available to more scientists and businesses. This may have major implications to the fine-chemical, pharmaceutical, petroleum, biotechnology industries etc. Not only does many studies suggest a higher reaction efficiency and yield, but also a drastic decrease in solvent use and lower costs if nanobiocatalysis fulfills its foreseen future.

This thesis will therefore focus on the feasibility of enzyme immobilization on nanomaterials and nanoparticles. A brief introduction to biocatalysis will be given as well as an overview of some relevant nanoparticles and nanomaterials that have shown potential for the purpose of immobilizing enzymes modifying the coveted features.

A simple explanation of enzymes and biocatalysis will be given. A superficial overview of the nanoparticles and nanomaterials that have piqued the interest of chemists with respect to nanobiocatalysis will also be given. This is to give some perspective for the discussion on nano-bio-catalytic processes.

The scope of this thesis will be limited to the use of nanomaterials or nanoparticles for immobilizing enzymes, further utilizing them for chemical catalysis. Meso- and micro-porous metal-organic frameworks will not be discussed. The use of whole cells as biocatalysts is not within the scope of this thesis, and will also not be discussed.

It seems relevant to discuss whether or not the use of nanomaterials and nanoparticles provides satisfying results regarding, yield, ease of catalyst removal, sustainability and environmental concerns, and economic aspects when used to immobilize enzymes. It is important that this method provides better results than existing methods to be feasible in the future. Environmental and sustainability concerns is important and will play a significant role in the assessment of feasibility. Eventually it is also important that the method is not overwhelmingly expensive as this will make it difficult to put in use commercially. However, environmental and sustainability aspects may be the most significant factor.

2 Theory

2.1 Enzymes

Enzymes are proteins (except ribozymes) that acts as biological catalysts.[6] They are made up of a sequence of amino acids and are relatively large molecules (macromolecules).[7] The proteins structure is held together by different forces.[8] The amino acids in the amino acid sequence is connected through covalent peptide bonds as shown in figure 2.1. The amino acid sequence is referred to as the primary structure of a protein. The different amino acids have different side groups (fig. 2.1). Some amino acids have non-polar aliphatic side groups, some are aromatic, some are polar, and some are polar with a formal charge. These side groups gives the amino acids dif-

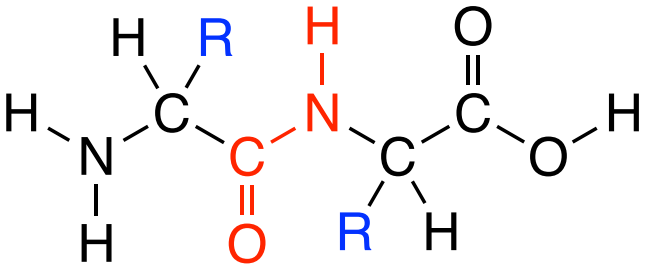


Figure 2.1: Two amino acids joined together through a covalent peptide bond. Peptide bond shown in red and side groups shown in blue.

ferent properties and different affinities to each other and the surrounding solvent and solutes. The chain of amino acids may fold in on itself and form the secondary and tertiary structure of a protein – their spatial shape (example shown in figure 2.2). This happens due to several types of bonds between amino acid side groups or between side groups and solvent. These interactions may be ionic bonds, hydrogen bonds, disulphide bonds between two cysteines (a polar uncharged amino acid), hydrophobic interactions between side groups, or hydrophilic interaction between side groups and solvent.

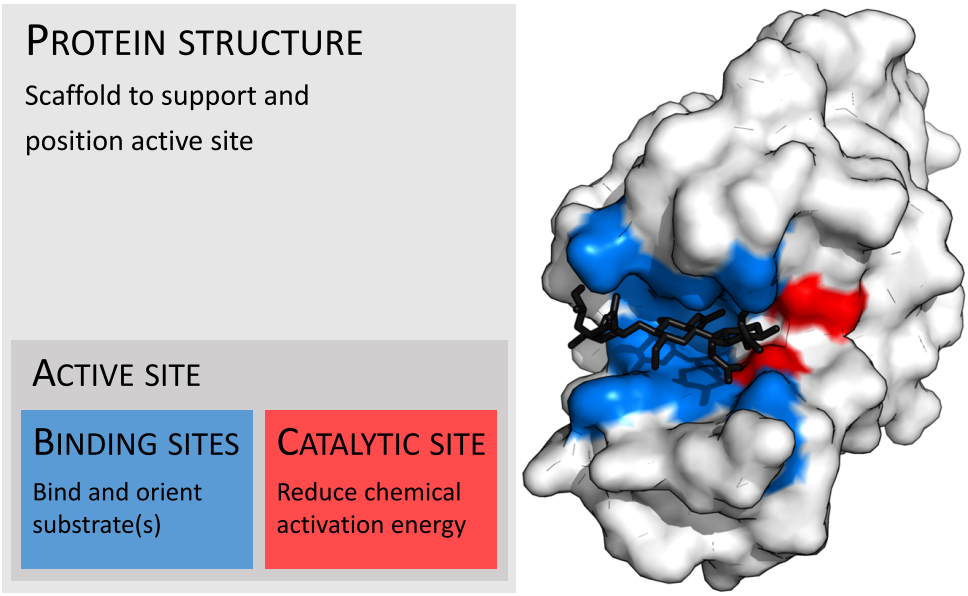


Figure 2.2: An enzyme with substrate. Catalytic site shown in red and binding sites shown in blue. Thomas Shafee, CC BY 4.0, via Wikimedia Commons.

The composition of amino acids in the amino acid sequence in a protein finally deter-

mines the proteins structure.[7] In an aqueous solution the polar parts of the amino acid chain tends to orient towards the outside of the protein due to their hydrophilicity.[9] On the other hand the hydrophobic parts of the protein tends to fold themselves towards the inside of the protein where they can avoid water. Therefore the solvent may influence the structure of the enzyme and thereby its catalytic properties.

It is shown that enzymes in aqueous-organic mixtures tends to denature (lose their structure and function), but in dry organic solvents or solvents with little water, enzymes often retain their structure and show a continued, or just slightly enhanced performance.[1] This for the better or worse. It is possible to exploit this trait.[1]

The reaction rate of most enzymes is dependent of the solution pH.[9] If one assumes that a an enzyme has two ionizable groups where only one of them is part of the catalytic cite, an increase in pH will deprotonate first one of the groups, forming the active enzyme. Further increasing the pH will eventually deprotonate the remaining groups, forming an inactive catalytic site. The enzyme will thus only be active within a certain pH-range, depending on those specific catalytic cites' pKa. This is a very basic model explaining the pH-dependency of the efficiency of enzymes. Different enzymes are active within varying pH ranges. Some enzymes show higher efficiency in alkaline solutions, and some are more effective in acidic environments. The width of the interval that shows good catalytic properties may also vary between different enzymes. Some enzymes are effective within a broad range of pH, while some only show catalytic activity within a more narrow range of pH.

Enzymes' catalytic activity is also dependent on temperature.[10] Essentially there is two factors influencing the catalysed reaction velocity. As with normal chemical reactions the reaction rate increases with increased temperature. This has, in theory, no limitations. However, above some temperature, the catalytic activity will start to decrease due to denaturation of enzymes. This is shown in equation (2.1) where E_{act} represents the active enzyme and X represents the denatured enzyme.

$$E_{act} \longrightarrow X \tag{2.1}$$

Denaturation is when the enzyme becomes unfolded and no longer retains its catalytic

activity. The temperature at which the enzymes begins to denature varies depending on the specific enzyme. Many enzymes begin to denature just above the physiological temperature (37 °C), while some denature at lower or higher temperatures. This is how the temperature dependency of enzyme activity traditionally is described. Later it has been developed a model, the Equilibrium Model, which better describes the observed behaviour of enzymes.[11] The model suggests that there is an equilibrium between the active enzyme and the inactivated version of the enzyme $\left(E_{inact}\right)$. At elevated temperatures the enzyme undergoes a reversible inactivation before the irreversible denaturation occurs as shown in (2.2).

$$E_{act} \rightleftharpoons E_{inac} \longrightarrow X \tag{2.2}$$

Only a small portion of the enzyme's structure actually participate in the catalytic activity.[9] Enzymes have a binding site and a catalytic site. The binding site catches the substrate and aligns them to the catalytic site where the desired reaction has a significantly lower activation energy. Together the binding site and the catalytic site constitutes the active site (figure 2.2). The rest, and most, of the enzyme acts as a scaffolding ensuring the geometrical configuration of the active site. Not just any substrate can fit into the binding site thus enzymes are selective and may react only with a few select molecules or groups. Enzymes are also specific and generally only do one kind of reaction on a certain substrate. There is, however, a practically infinite number of combinations of amino acid sequences, and there is consequently a potential enzyme for every substrate and every reaction. It's just a matter of finding/making them.

2.2 Biocatalysis

Biocatalysis is the use of biological systems, organisms, or enzymes to catalyse chemical reactions.[12]

Biocatalysis have been in use since before recorded history, in the production of alcoholic drinks, cheese, and soy-derived food.[12] This without any knowledge of enzymes. The ancient applications of enzymes may best be described as an art, rather than tech-

nology as the people using biocatalysis had no knowledge of enzymes or their ways of function.

The first acknowledgement of an enzyme, more specifically diastase, as a catalyst was by Berzelius in 1835.[13] Diastase was later utilized in the brewing industry building on the findings of Payen and Persoz.[13,14]

Emil Fischer investigated different enzymes and whether they hydrolyzed certain saccharides or not.[15–17] He found that some enzymes hydrolysed one anomer of a sugar, but not the other. This turned out to be true for several enzymes as well as several substrates. These are the findings that led Fischer to derive his theory on specificity for enzymes.

Eduard Buchner, in 1897-1898, published a series of papers about experiments on so-called "fermentation" (in which they meant enzymatic activity).[12,18] He extracted press juice from yeast cells and filtrated it under elevated pressure and then re-filtrated it. In the presence of chloroform, an antiseptic, this substance supposedly produced carbon dioxide from some sugars, but not lactose and mannitol. This was contrary to previous belief that processes in living organisms – where alcoholic fermentation was the most prominent example – was due to something more than a pure chemical/physical process. This also helped verify the key-in-lock theory proposed by Fischer.[15,19] Several scientists replied to this publication and challenged Buchner's work.[12] This "vis vitalis" consensus in the scientific community was therefore challenged and this period is important as it show a paradigm shift over to a understanding that all such reactions was purely chemical processes without any other hidden biological forces.[12]

Biocatalysis is in use today in a variety of industries.[5,20] Examples of such industries may be the pharmaceutical industry,[21] production of (bio-)fuels and the fine-chemical industry[22,23] as well as the traditional food and detergent industry.[20,24,25] These are illustrated in figure 2.3.

Biocatalysts have shown to be useful in green chemistry as enzymes holds excellent

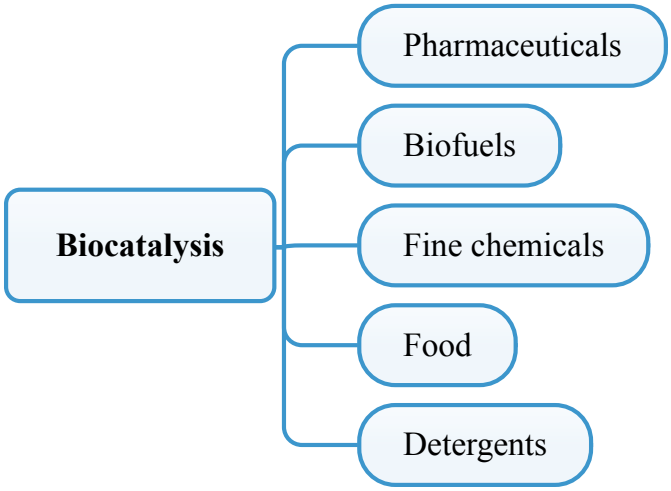


Figure 2.3: Graphic representation of some industrial applications for biocatalysis.

regio- and stereo-specificity as well as the use of water as solvent under ambient conditions lead to a greatly reduced usage of solvent and energy.[26]

2.3 Immobilizing enzymes

Immobilizing enzymes means confining them to another material or support other than the substrate.[6] This will alter the mobility of enzymes. They will be connected to another material so that they do not move around in a solution,[5] or they may get an altered conformational flexibility. This may enable their safe recovery and reusability.[27] This can give them a more stable structure or change their catalytic activity as well ass modify the enzymes' sensitivity to changes in salinity, pH, temperature, and solvents.[20,28]

There are several ways to immobilize enzymes to a support. A graphical overview is given in figure 2.4.[28]

2.3.1 Binding to a support

Enzymes may be bound directly to a support matrix by weak or strong bonds.[28] Forces such as van der Waals and hydrophobic interactions represent weak forces and ac-

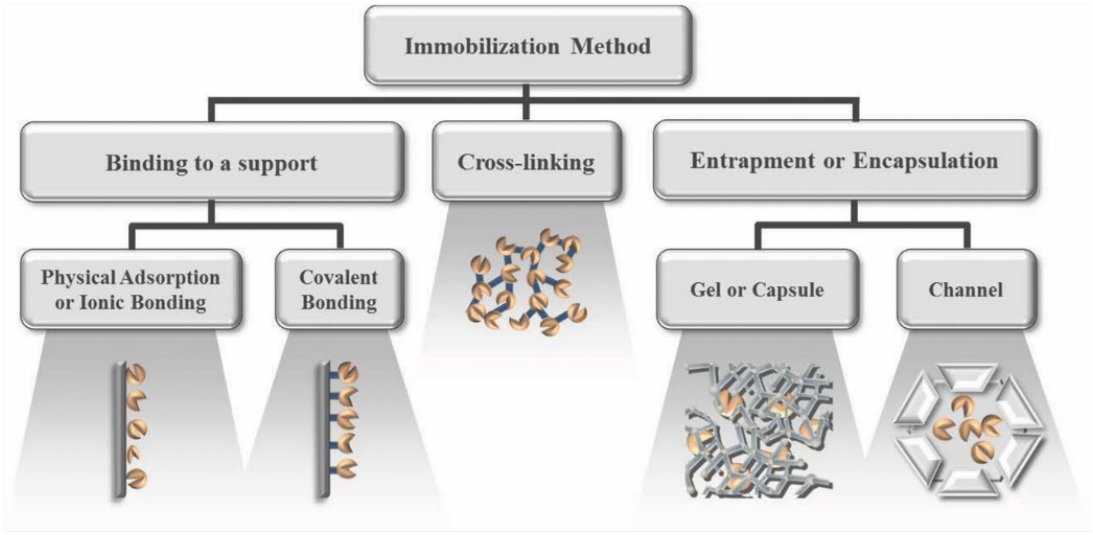


Figure 2.4: A graphic representation of the various methods for immobilizing enzymes.

counts for the adsorption of enzymes to supports. Weak forces are generally known to be too weak for realistic industrial conditions. Ionic bonds are stronger, and covalent forces are the strongest.[3] Sufficient bond strength keeps the enzyme confined to the support. For feasibility of direct bonding to a support it is crucial that such bonding does not deactivate the enzyme irreversibly or alter the other enzymes' properties in such a way that the immobilization becomes impractical.

2.3.2 Cross-linking

Enzymes may also be cross-linked.[28] A bifunctional reagent may be used to connect several enzymes together. This involves reactions between the amine groups on lysine (an amino acid) residues at the enzymes' external surface and glutaraldehyde () or other bifunctional aldehydes forming amine linkages. This often leads to a decrease in catalytic activity.[5] It has been shown that larger bulky polyaldehydes retains a much larger fraction of the immobilized enzymes' activity due to their inability to penetrate into the enzymes' active site and react with the amino acids necessary for catalytic activity.[29] Cross-linked enzyme aggregates are practically insoluble,[28]

and this makes them easy to separate out from the reaction mixture and potentially reuse. Precipitating cross-linked enzyme aggregates out of solution removes the need for very pure enzymes, and may consequently reduce the total production cost.

2.3.3 Entrapment or encapsulation of enzymes

Another method for immobilizing active enzymes is entrapment or encapsulation.[28] The enzyme is physically restricted within a confined space or polymer matrix.[30,31] Examples of such a network can be a silica sol gel, an organic polymer or microcapsules.[28] There are some concerns regarding lowered mass-transfer and enzyme loading capacities when it comes to encapsulation and entrapment. Limited mass transfer and enzyme loading capacities may lead to a lowered catalytic activity when compared to the free enzymes. Most entrapped-enzyme-systems require additional linking between enzyme and entrapment matrix as the pure physical restraints are too weak to hinder enzyme leakage.[28,32] Generally, the entrapment matrix must be synthesised in presence of the enzyme. Sol gels, as inorganic entrapment matrices, shows customizability as several measures and variations can be done during the synthesis and entrapment process that can affect parameters such as mass transfer, loading capacity, heat resistance, reusability, etc.[28,31] For example, introduced organic functional groups to the otherwise inorganic matrix may form covalent bonds to enzymes and tether them to the entrapment matrix.

2.4 Nanobiocatalysis

2.5 Nanomaterials and nanoparticles

According to ISO/TS80004[33] a nanomaterial is a "material with any external dimension in the nanoscale or having internal structure or surface structure in the nanoscale" where nanoscale is defined as "length range approximately from 1 nm to 100 nm". The European Commission[34] have defined nanomaterials as "*A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size*

distribution, one or more external dimensions is in the size range 1 nm - 100 nm. *In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %.*" These definitions include a wide range of materials. Such materials may be fullerenes (including "buckyballs" and nanotubes), titanium dioxide (TiO_2)[35] and many other materials and particles. The possibilities for new nanomaterials seems endless.

Nanomaterials are used in several different industries today and some of them include cosmetics industry,[35] sports industry,[36] agriculture,[37] oil and gas industry,[38] and in the medical field.[39] This is illustrated in figure 2.5.

The field of chemistry has many potential applications for nanotechnology.[40] Many of those applications being in catalysis. Nanoparticles and nanomaterials are appreciated due to high surface-to-volume ratio, optical-, and magnetic properties. Some nanoparticles may exhibit superparamagnetism which is a very desirable feature in some contexts.[41] Nanomaterials may be used in hydrogenation reactions, oxidation reactions, cross-coupling reactions, C−N bond formation reactions and cyclization reactions[42] as shown in figure 2.5.

Nanoparticles may be divided into two categories – magnetic and non-magnetic nanoparticles.[43]

2.5.1 Magnetic nanoparticles

Magnetic nanoparticles may be used as bare catalysts[44] or as an immobilizing agent for other catalysts such as enzymes.[45] They are appreciated for their low toxicity, easy of surface modification, reusability and large enzyme capacity. They are especially appreciated because they are easy to separate out from the reaction medium through the application of an external magnetic field.[31,46] Enzymes immobilized on magnetic nanoparticles shows great stability allowing them to be stored and reused several times.[31] Their small size enables them to immobilize many enzymes per unit mass of particles and gives them good binding efficiency.[47] Examples of magnetic

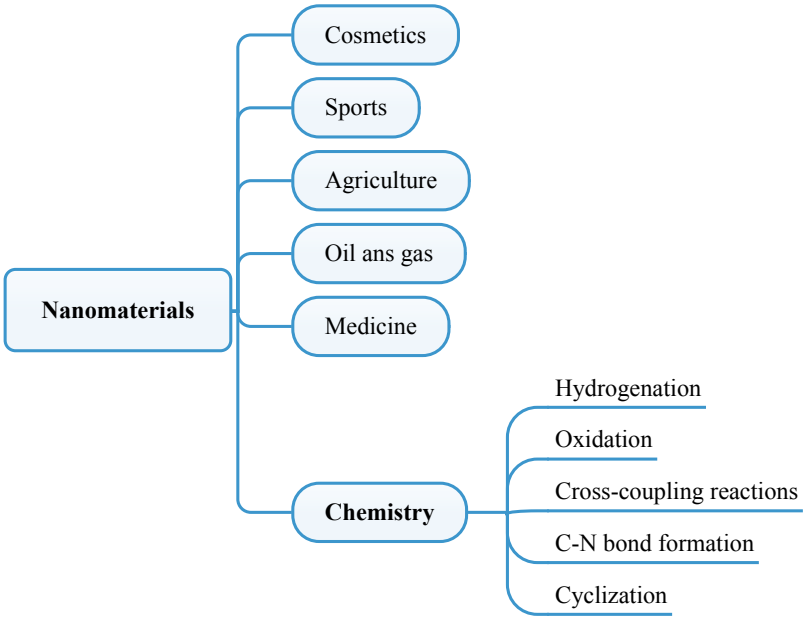


Figure 2.5: Graphic representation of some industrial and chemical applications for nanotechnology.

nanoparticles is magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) which are widely used because of their low toxicity, availability, superparamagnetic properties and low environmental impact.[43,45] Magnetic nanoparticles have some disadvantages as well. Due to magnetic dipole-dipole interactions between them, many particles may aggregate and form larger particles. Fe_3O_4 particles may sometimes also oxidize and this may be a disadvantage when upscaling to industrial proportions. These limitations may be overcome by modifying their surfaces with various materials.[47]

2.5.2 Non-magnetic nanoparticles

The use of non-magnetic nanoparticles is increasing and has applications within several industries, including chemistry, biotechnology, biomedicine, electrochemistry, and pharmacy.[43] Nanoparticles must have low toxicity and preferably have a low production cost. Carbon nanotubes, titanium oxide, platinum, semi-conductive materials, silver, gold and other materials have been used to make non-magnetic nanoparticles.[20] Non-magnetic nanoparticles can be either *soft* or *hard*.[48] Soft nanoparticles are non-ceramic and non-metallic, such as polymers and lipid-based particles. The term hard

nanoparticles includes all ceramic and metal nanoparticles.

2.5.3 Nanofibers

Nanofibers are slender thread-like entities with a cylindrical shape.[28] Nanofibers can be considered as nanomaterials thanks to their nano-range diameter dimension that may be under 100 nm. There are several manufacturing and recovery methods for nanofibers, with electrospinning being the most typical choice due to the good control of fiber dimensions, scalability and repeatability as well as the wide variety of polymers available for fabrication. Nanofibers stand out among other nanomaterials because of their ease of manufacturing and ease of recovery. Nanofibers show great properties regarding enzyme immobilization ensuring their continued use in biocatalysis.[28] Enzymes may be encapsulated in nanofibers by coelectrospinning a polymer with enzymes creating homogeneous enzyme-encapsulated nanofibers that are highly bioactive.[49]

2.5.4 Nanotubes

Carbon nanotubes are graphitic sheets rolled up into a cylindrical shape with diameters up to 100 nm.[50] Lengths are in the micrometer scale. Carbon nanotubes are biocompatible and exhibit extraordinary electrical, thermal, and mechanical properties. Carbon nanotubes electrical properties have proven useful i biosensors giving higher sensitivities, lower detection limits and faster electron transfer kinetics. Carbon nanotubes may be single walled, consisting of a single graphitic tubule, or be multi walled having several graphite layers surrounding a single tubule. Enzymes may be attached to carbon nanotubes by either covalent bonding or non-covalent adsorption to the tube wall exterior. Compared to covalent bonding the adsorption method is considered the most promising due to the preservation of enzyme conformation during adsorption. Adsorption of enzymes to carbon nanotubes may be done by direct adsorption using predominantly hydrophobic and aromatic $\pi-\pi$ interactions, polymers, biomolecules, or surfactants. Covalent binding of enzymes to carbon nanotubes may be done using linking molecules or reacting free amine groups on to enzyme to carboxylic groups on

the surface of the nanotube with carbodiimide.[50,51] Enzymes immobilized on carbon nanotubes are shown to retain a high fraction of the activity of their free counterparts as well as improved resistance to high temperatures.[52]

2.5.5 Nanoflowers

Nanoflowers have piqued the interest of researchers due to their surface-to-volume ratio and facile synthesis.[53,54] Enzymes immobilized on organic-inorganic hybrid nanoflowers have shown great increase in catalytic activity, stability, and durability when compared to their free counterparts. This has been attributed to the lowered mass-transfer resistance as nanoflowers have an open shape that does not hinder the access of substrates to the immobilized enzyme. Nanoflowers was discovered by accident in 2012 when Ge, Lei, and Zare[55] added $CuSO_4$ to a phosphate buffered saline with bovine serum albumine. A typical synthesis method for nanoflowers are adding metal-salts to a phosphate buffered saline containing enzymes.[53] The amide-groups of the protein provides nucleation sites for the crystals of the phosphate salt to start growing into petals forming the flower-like structure. The nanoflowers eventually precipitates out of solution. Scientists have made nanoflowers using different enzymes and different metal cations such as Ca^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} and more.[53]

2.6 Assessing sustainability/environmental impact/"greenness" of reactions

The terms "sustainable", "environmentally friendly/hostile" and "green" can be quite vague and ambiguous. There have been some attempts to determine what makes a chemical process good for the environment, sustainable and green. Especially two models have been developed and are often referred to when assessing how good or bad a reaction is for the environment. The twelve principles of green chemistry and the E-factor.

2.6.1 Green chemistry

Green chemistry is a concept developed in the early 1990s.[56] The definition of green chemistry is[56] "design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances." Twelve principles were developed in 1998 by P. Anastas and J. Warner that say something about how one should strive to design new synthesis pathways. These principles are shown in figure 2.6.

2.6.2 E-factor

The E-factor proposed by Roger Sheldon[57] provides a method of assessing the environmental impact[58] of a chemical reaction. This method takes into account several parameters such as solvent usage and waste generation.

1. **Prevention**. It is better to prevent waste than to treat or clean up waste after it is formed.
2. **Atom Economy**. Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.
3. **Less Hazardous Chemical Synthesis**. Whenever practicable, synthetic methodologies should be designed to use and generate substances that pose little or no toxicity to human health and the environment.
4. **Designing Safer Chemicals**. Chemical products should be designed to preserve efficacy of the function while reducing toxicity.
5. **Safer Solvents and Auxiliaries**. The use of auxiliary substances (e.g. solvents, separation agents, etc.) should be made unnecessary whenever possible and, when used, innocuous.
6. **Design for Energy Efficiency**. Energy requirements of chemical processes should be recognized for their environmental and economic impacts and should be minimized. If possible, synthetic methods should be conducted at ambient temperature and pressure.
7. **Use of Renewable Feedstocks**. A raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable.
8. **Reduce Derivatives**. Unnecessary derivatization (use of blocking groups, protection/ deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste.
9. **Catalysis**. Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.
10. **Design for Degradation**. Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment.
11. **Real-Time Analysis for Pollution Prevention**. Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.
12. **Inherently Safer Chemistry for Accident Prevention**. Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires.

Figure 2.6: The twelve principles of green chemistry. Reproduced from ref. [56] with permission from the Royal Society of Chemistry.

3 Discussion

Searching for "biocatalysis", "nanomaterials" or "nanobiocatalysis" yields vast amounts of research papers in the thousands. The literature used as a basis for this thesis is therefore mainly review articles and a few research papers. The subject of nanobiocatalysis appears at first glance to be a field of great interest to both academia as well as the industry and a field where a lot is happening.

The concept of immobilized biocatalysts have been around for a while.[12] The use of immobilized enzymes in chemistry have been somewhat limited mainly due to lowered catalytic activity of the immobilized enzymes.[4] Recent development and emerge of novel synthetic nanomaterials[42] have driven attention to nanomaterials as promising immobilizing supports.[20] Results so far are promising, but there are limited number of nanocarrier-immobilized biocatalysts that have been successfully reused more than 10-15 times. These nanobiocatalysts are still at a proof-of-concept stage in a laboratory scale, and there are still some challenges that have to be overcome to achieve real-time industrial scale applications. One of these challenges are cost.

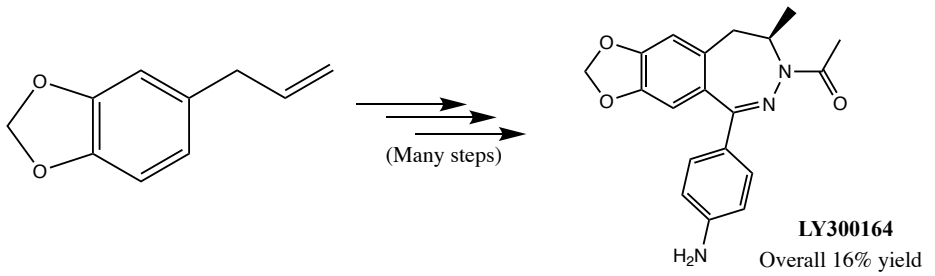
There is a myth spooking around the catalysis community about enzymes being inherently expensive.[58] However, if produced in in large quantities, the price of enzymes can be well below 200 Euro per kg, which is reasonable, according to Torello et al.[58] However, the cost of immobilized enzymes may be much greater. An immobilized enzyme may cost 10-fold as much as the free enzyme.[58] Therefore the use of immobilized enzymes on an industrial scale is rather modest simply because the cost of enzyme immobilization is not worth the return from enzyme reuse.[59]

The cost of nanobiocatalysis still seems to be too great to catch the interest of the commercial industry. However, since the robustness and thus reusability of immobilized biocatalysts has improved with the implementation of nanosupports, the overall cost of the end product may eventually decrease.[20] It seems that the technology is still not quite there, since there is little industrial utilization of this technology to bee seen out there yet. Thus there is still some improvements that needs to be done to reduce the cost of nanobiocatalysts. The development of nanotechnology, however, may lead to

the manufacturing of nanomaterials at lower costs in the future, and the potential for highly profitable methods within nanobiocatalysis is definitively present. The importance of the sustainability and "green" aspect may, however, increase and surpass the importance of economic aspects in the future thus weakening the importance of cost in the future. The cost is nevertheless undeniably important and it is important to keep a constant focus on cost in future research, facilitating interest from private investors further advancing innovation in the industry.

Biocatalysis is viewed by many authors as being the pure definition of green chemistry. This is often attributed to the mild reaction conditions associated to enzymes such as near to physiologic solvent (water), pH and temperature. The biocatalysts are also considered to be non-toxic and non-hazardous to the environment thus not possessing much disposal difficulty.

This is very prominent in the pharmaceutical industry. Synthesis of pharamceuticals is often accompanied by a large consumption of organic solvents.[26] This due to low yield and many reaction steps. An example set forth by Ran et al.[26] is the synthesis of LY300164 (Talampanol), a drug investigated for treating epilepsy and neurodegenerative diseases. The chemoenzymatic approach (3.2) had an overall yield of 51% and the old synthesis pathway had an overall yield of only 16% (3.1).



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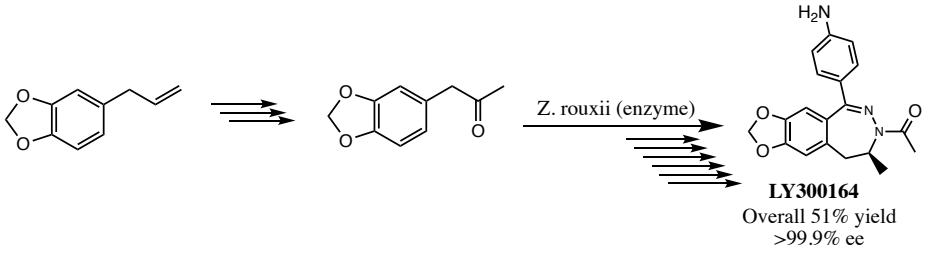

(3.2)

The chemoenzymatic approach also eliminated the use of transition metal oxidants

and reduced the consumption of organic solvents by 340 000 L for every 1000 kg of LY300164 produced.

There seems to be a broad unity in the scientific community about biocatalysis being environmentally friendly. The factor often used to discuss whether a certain synthesis is environmentally friendly or not is the principles of green chemistry.[56] Nanobiocatalysis is in many cases argued to be in line with many of the principles of green chemistry and thus environmental friendly. There is, however, some opinions[26] that a mere qualitative discussion of a given method is not enough to assess if one synthesis is more environmentally friendly than another. It is argued that a quantitative comparison against reference process is necessary to make any certain conclusions about a certain process's "greenness". A full life cycle assessment can be extensive and tedious to perform. Although there is some discussion about the topic, there seems to be quite a broad understanding among the scientific community that biocatalysis often possesses potential to be a more environmentally sustainable synthesis pathway than the "classical" chemical synthesis procedures.

The E-factor is also used to assess the environmental impact of chemical reactions[57,58] procedure. Torello et al.[58] argues that the E-factor is a very convenient and simple tool for comparing a given reaction system with a reference system. The E-factor can consequently be used for determining the sustainability of a nanobiocatalytic synthesis compared to the traditional chemical pathway.

The assessment itself for determining whether nanobiotechnology is a "greener" synthetic route than older syntheses is difficult to do as there are many parameters in play. These parameters also vary with the different nanomaterial, immobilization methods and more. For that reason, it is not manageable to attempt on such a definite assessment in this thesis. However, since there is a quite good consensus about biocatalysis being a sustainable and environmentally friendly synthesis, the development of nanoparticles and nanomaterials with the same characteristics can lead to many green/eco-friendly nanobiocatalytic application thus driving the relevant industries into more environmentally sustainable productions.

Some bare nanomaterials and nanoparticles have shown to also have catalytic properties by themselves.[42,60] Thus there is a potential for nanosupport-enzyme hybrids to have a combined or maybe even synergistic catalytic effects.[61]

It is indeed possible to conclude from literature[20,58,62,63] that immobilization of enzymes onto nanoparticles and nanomaterials is a feasible alternative to classical organic synthesis. This especially considering both overall yield and the savings in respect to organic solvent usage and metallic catalysts. Enzymes immobilized on nanosupports generally show clearly improved stability and robustness. This allows several reuses, an may in turn make the overall synthesis cost lower even though the enzyme immobilization itself may be costly. It is generally recognized that nanosupports for enzyme immobilization have properties that make them easy to separate out of solution.[20] For example cross-linked enzyme aggregates becomes practically insoluble,[28] and hence are possible to simply filter out of solution.[46] Another example is magnetic nanoparticles that are especially highlighted for their ease of separation from reaction mixture by applying an external magnetic field.[46] Enzymes do not unconditionally perform better when immobilized on nanostructures. The performance of immobilized enzymes is highly dependent on the enzyme itself as well as the nanosupport. Some studies found decreased or total loss of kinetic activity after immobilization.[5] Immobilization of enzymes on nanostructures may result in a lowered catalytic activity, but this is in many cases acceptable as immobilized enzymes exhibit improved storage stability, an can be reused several times, which may give them applicability in industrial processes.

4 Conclusion

The use of nanomaterials for immobilizing enzymes possesses great potential, and with the continued development of research and technology the prospect of nanobiocatalysis seems very promising. Nano-immobilized enzymes generally possesses good properties regarding catalytic activity, ease of recovery and reusability.

Biocatalysis is generally considered green and environmentally friendly. Immobilized enzymes are costly to produce and there is a limited application on an industrial scale. This limited industrial utilization of nanobiocatalysis is attributed to the high cost of immobilized enzymes compared to the inferior free enzymes that cannot be reused. It is expected that the development of nanotechnology will lead to a lower production cost in the future, and therefore nanobiocatalysis have the potential to becoming the environmentally friendly alternative as well a profitable production method. Some nanomaterials and nanoparticles even inhibit catalytic by themselves, and some nanobio catalysis systems may benefit from combined catalytic effects. As the environmental aspect may become the ever more important in the future, nanobiocatalysis have a potential for becoming an important technology in the future of chemical synthesis.

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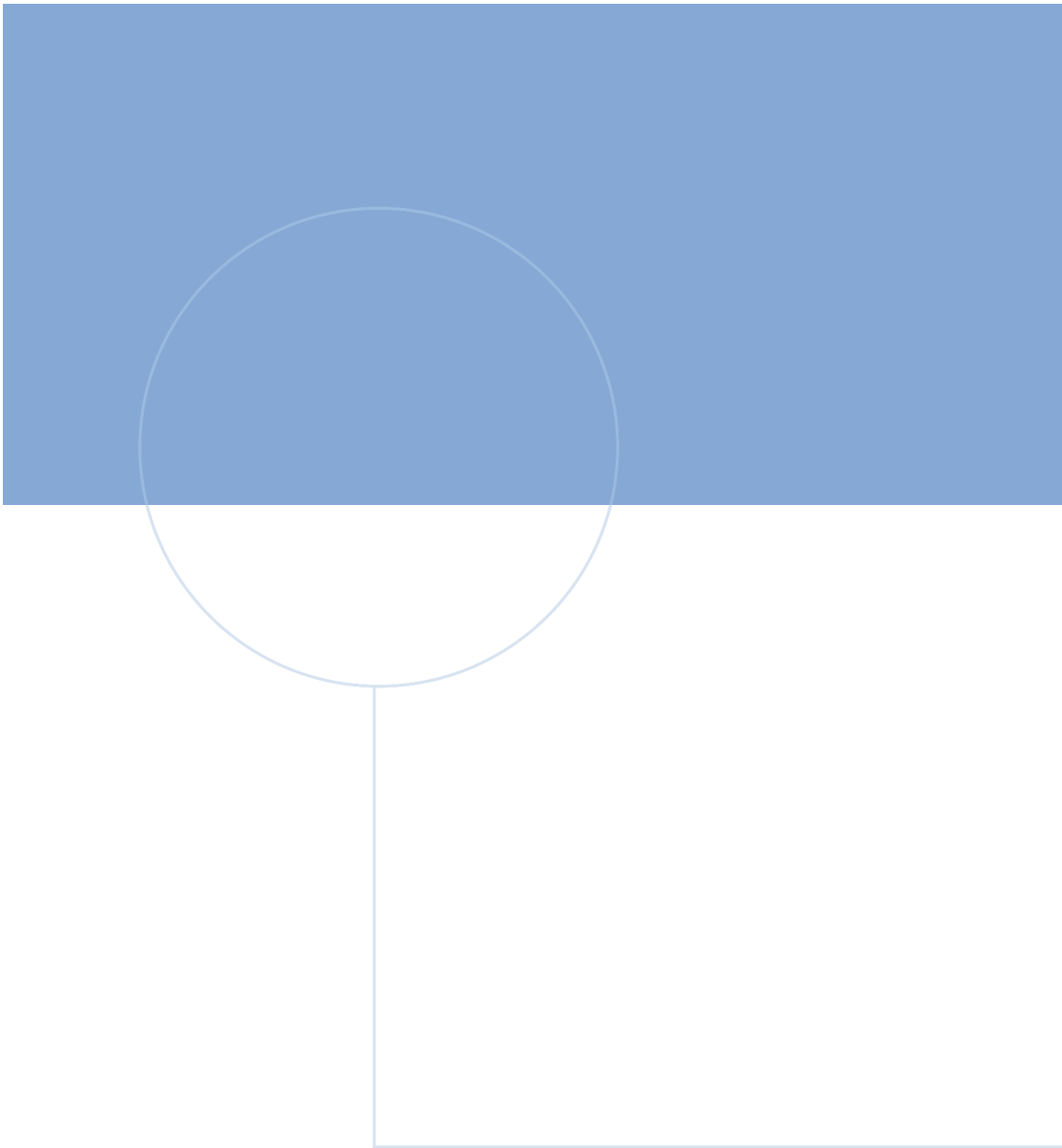

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