

Design of heterogeneous biocatalysts
for flow chemical processes
Towards a greener transamination reaction

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degree of Doctor of Philosophy in Agricultural Sciences and
Biological Engineering

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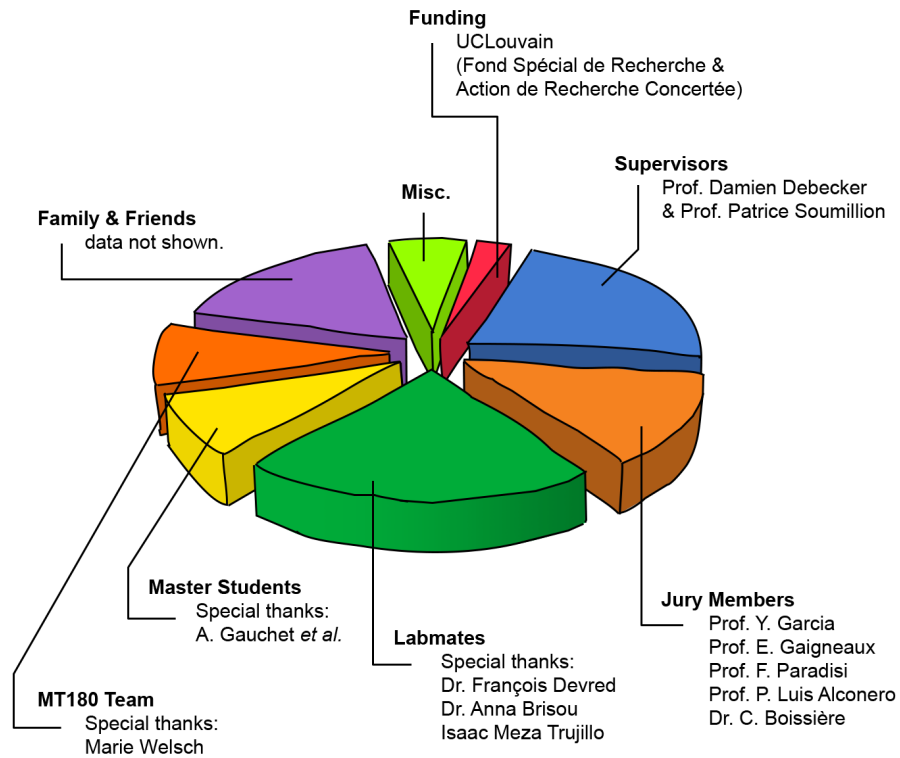


Figure 0 – Acknowledgements. To all of you, and to everyone I have forgotten. This work would have been tasteless without all of you. Thank you for your precious help and the good times.

List of communications

Papers

The first chapter of this work has been published in [1] :

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Table of Abbreviations

AAAL – Aspartic acid ammonia-lyase
ABA – 3-DL-Aminobutyric acid
AL – Ammonia-lyase
AFEI – Adsorption facilitated by electrostatic interactions
API – Active Pharmaceutical Ingredients
APTES – 3-Aminopropyl-triethoxysilane
ATA – Amine transaminase
ATA-117-11Rd – Mutated version of ATA-117
ATR-FTIR – Attenuated Total Reflectance Fourier-Transform Infrared Spectroscopy
BAP – 4'-Bromoacetophenone
BMBA – 4-Bromo- α -methylbenzylamine
CALB – *Candida antarctica* Lipase B
CLEA – Cross-Linked Enzyme Aggregates
CLEC – Cross-Linked Enzyme Crystals
Coum, Me-Coum, Et-Coum – Coumaric acid, coumaric acid methyl ester and coumaric acid ethyl ester
CSTR – Continuous Stirred Tank Reactor
DMSO – Dimethylsulfoxide
dTG – Differential thermogravimetric analysis
EC – Enzyme Commission number
FAD – Flavine Adenosine Dinucleotide
FBA – 2-Formylbenzylamine
FBR – Fixed-Bed Reactor
FDH – Formate Dehydrogenase
FPA – (4-Fluorophenyl)acetone
GC – Gas Chromatography
GDH – Glucose Dehydrogenase
GLYMO – 3-Glycidyloxypropyl-trimethoxysilane
HEwT – *Halomonas elongata* omega-transaminase
His-Tag – Poly-Histidine Tag
HPLC – High Performance Liquid Chromatography
IPTG – Isopropyl β -D-1-thiogalactopyranoside
IR – Infrared spectroscopy
ISPR – In Situ Product Removal
JOSIPHOS – Chiral ligand containing disphosphine and iron atom
LDH – Lactate Dehydrogenase
Lys-NTA – N α ,N α -bis(carboxymethyl)-L-lysine
MIO – 3,5-Dihydro-5-methylidene-4H-imidazole-4-one
MTES – Methyltriethoxysilane

NAD / NADH – Nicotinamide Adenine Dinucleotide (H for reduced form)
 NTA:Ni, NTA:Co – Nitrilotriacetic acid loaded with Ni²⁺ or Co²⁺ ions
 OTMS – Octyltrimethylsiloxane
 PDB – Protein Data Bank
 PDMS – Polydimethylsiloxane
 PFR – Plug-Flow Reactor
 PISA – Proteins, Interfaces and Structures Assemblies
 PLP – 5'-Pyridoxal phosphate
 PMP – Pyridoxamine phosphate
 PTFE – Polytetrafluoroethylene
 RSD – Relative standard deviation
 SEM – Scanning Electron Microscopy
 Si(HIPE) – Silica High Internal Phase Emulsion
 SLM – Supported Liquid Membrane
 TA – Transaminase
 TAL, and FjTAL – Tyrosine Ammonia-lyase (from *Flavobacterium johnsoniae*)
 TEOS – Tetraethylorthosilicate
 TGA – Thermogravimetric Analysis
 THF – Tetrahydrofuran
 TTAB – Trimethyltetradecylammonium bromide
 TYR, Me-TYR and Et-TYR – Tyrosine, tyrosine methyl ester and tyrosine ethyl ester
 YADH – Yeast Alcohol Dehydrogenase

Summary

Given the need to produce organic compounds in a greener way, transaminases (TA) have been used for the green production of chiral amines (that are precursors of many bioactive compounds). There are two main limitations to the use of TA: the price of enzymes, and the unfavourable thermodynamics (that favours the amine deamination). Immobilizing TA allows to recover the biocatalyst and to reuse it in further transformations. Working in flow mode allows to increase the overall process productivity. Strategies to displace equilibrium (based on Le Chatelier's Principle) allow performing the asymmetric synthesis (*i.e.* the production of chiral amine of industrial interest).

The main objectives are: (i) developing suitable carriers for enzyme immobilization (control on shape and surface composition); (ii) finding an optimal method for enzyme immobilization (control on bounds between enzyme and carrier) while retaining activity; (iii) assessing biocatalysts efficiency in flow mode; (iv) the development of strategies to displace equilibrium towards the asymmetric synthesis. At the end, we wanted to develop a standard method (on immobilization and flow reaction) that could be applied to any enzyme or reaction.

Enzymes are first studied in batch mode, in the kinetic resolution way. Several enzymes are used (different types of TA, and other enzymes –tyrosine ammonia-lyase, TAL, useful for organic synthesis). Several immobilization methods are investigated (simple adsorption, covalent grafting, His-tag immobilization, etc.). Porous silica monoliths -Si(HIPE)- are employed as solid carriers suitable for flow transformations. Carriers are characterized (SEM, IR, TGA, etc.). Biocatalysts are systematically tested in flow mode to assess enzyme activity.

In the First Chapter, we developed a suitable flow device for flow transformations. The enzyme is studied in batch and flow modes after several optimizations (analytical methods, reaction conditions). Transaminases (ATA-117) are active when immobilized onto silica monoliths through covalent grafting (monoliths functionalized with APTES (3-aminopropyltriethoxysilane) and pre-activated with glutaraldehyde). Simple adsorption led to poor activity. Enantioselectivity is retained; enzyme is stable over long period of time.

APTES functionalization was optimized in the Chapter II: a better dispersion, and higher repeatability and activity (when used as enzyme carrier) were obtained when using water saturated toluene and dry monoliths for APTES grafting. The influence of temperature on enzyme impregnation was highlighted. Imine reduction was studied in order to get even more stable biocatalysts.

Hydrophobicity of carriers was studied in the Chapter III. Two methods for the synthesis of hydrophobic monoliths were studied: the one-pot method led to unsatisfying results (brittle monoliths, hard control on morphology); the post-grafting method led to robust hydrophobic monoliths. However, hydrophobicity did not influence the flow biocatalysts activity.

In the Chapter IV, a His-tagged version of ATA-117 (His-TA) was produced with high purity. Immobilization through covalent grafting (epoxide or APTES/glutaraldehyde) and His-tag were studied. Best result (maximal yield in flow mode) was obtained when His-TA was immobilized on APTES grafted monoliths (in combination with glutaraldehyde).

Research for finding efficient method for equilibrium displacement was the main topic of the Chapter V. Mono and multi-enzymatic methods were studied. After optimization (enzymes and substrates concentrations), a displacement strategy was selected for implementation in flow mode (pyruvate consumption with lactate dehydrogenase, and cofactor recycling with glucose dehydrogenase). Satisfying activity in flow was obtained for His-TA immobilized (on APTES/glutaraldehyde grafted monoliths), and used in the amination direction. ATA-117 and HEwT (another TA) were poorly active.

In the Chapter VI, we applied our strategy (flow transformations with immobilized enzymes on silica monoliths) to another kind of transformation (the tyrosine deamination) catalysed by another enzyme, the tyrosine ammonia-lyase (TAL). Using the same device and the best carrier developed for TA (APTES/glutaraldehyde grafted), immobilized TALs were highly active in flow mode.

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Introduction

1 Context

Since industrial era has begun, chemical industries have released large amount of hazardous compounds in the environment. They produced tons of toxic, polluting and non-recoverable by-products, using large quantities of reactants, solvent and energy. In particular, pharmaceutical industries are largely responsible for that issue: drugs must be produced in large amount and quickly to meet the consumer demand. As a highly pure product is generally needed, solvent for purification (de-racemisation, distillation) and polluting materials (chiral precursors, organometallic catalysts releasing toxic heavy metals) are employed in large quantities [2].

As chiral amines constitute the active pharmaceutical ingredient of many drugs, most of the largest pharmaceutical industries are active on the chiral amines market: Pseudoephedrine (Sinutab®, Johnson & Johnson), Sertraline (Zoloft®, Pfizer), Oseltamivir (Tamiflu®, Roche), Rivastigmine (Exelon®, Novartis), Clopidogrel (Plavix®, Sanofi), Sitagliptine (Januvia®, Merck), Lisdexamfetamine (Vyvanse®, Shire), Linagliptine (Tradjenta®, Boehringer-Ingelheim Pharmaceuticals), etc. Those highly consumed medicines act as decongestants, anti-depressants, antiviral drugs, treatments for neurological disorders, diabetes, etc. They are produced in abundance to ensure supplies to the global market. For instance, Clopidogrel – antiplatelet agent – is prescribed to more than 100 million people worldwide [3]; Sertraline – a treatment against neurological disorder – was prescribed to more than 37 millions people in USA in 2011 [4].

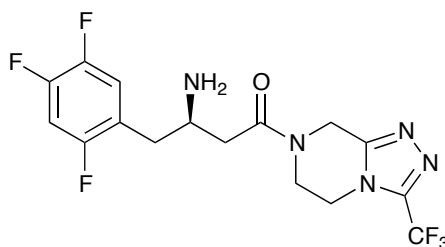


Figure I - 1 – R-Sitagliptine. It is an anti-diabetic drug. A recently developed engineered transaminase is now able to catalyse the synthesis of this compound.

In this context, following more closely the twelve principles of green chemistry [5] would help to reach more efficient processes for the chiral amine production: waste prevention, atom economy, less hazardous syntheses, safer solvents, design for energy efficiency, renewable feedstock, use of catalysts, etc. Green chemistry, as Matt Truppo (director at Merck's Process Chemistry Biocatalysis group) put it, is *"more than preventing waste; it's about increasing efficiency, reducing cost and operating in a more sustainable way"*. For decades, pharmaceutical industries have used homogeneous organometallic catalysts for chiral amines production [6]. Using enzymes for that purpose is a greener alternative and is increasingly studied: the greener production of Sitagliptine (Figure I - 1) by transaminases is now implemented at large scale [7,8]. Also, the production of Pseudoephedrine by transaminases was recently demonstrated [9].

Our project proposes to tackle the persistent major challenge: immobilizing the enzymes in order to obtain a sustainable and efficient biocatalyst and to make the biocatalytic process more realistic, opening perspectives for an application at an industrial scale.

2 State of the Art

2.1 Chiral amine production

Chiral compounds represent 56 % of active pharmaceutical ingredients (API) available on the current drug market [10]. We expect more than 95 % of API will be chiral in 2020 [11]. This is not surprising, given that all living organisms are built on chiral biomolecules: a drug target like a protein will never recognize R- and S-enantiomers of a molecule in the same way. We are essentially enantioselective organisms. For example, we synthesize our proteins strictly from L-amino acids [12].

Consequently, if a compound R or S is administrated, the physiologic effect will not be the same. The enantiomer that exhibits the desired positive properties is called eutomer, while the other enantiomer is called the distomer [13]. In some cases, the distomer exhibits weak positive properties (sometimes different from the eutomer properties), or is inert with respect to the biological organism. In the worst cases, the distomer is highly toxic.

For example, the R enantiomer of methamphetamine is a powerful decongestant, while the S-methamphetamine is a recreational drug [14]. The S-Penicillamine (Figure I - 2) is a chiral amine drug prescribed for the treatment of polyarthritis, but the R-Penicillamine is highly toxic [15]. Propoxyphen enantiomers both exhibit positive but different properties: (1S,2R)-enantiomer is a pain killer (Davron®), while (1R,2S)-enantiomer has antitussive properties [16]. For the anecdote, (1R,2S)-enantiomer is commercialized as Norvad®, *i.e.* the brand name Davron® written backwards...

Thus, it is obviously necessary to know the properties of each enantiomer before its use as a drug. Since 1992, the U.S. Federal Food and Drug Administration (FDA) forces the drug manufacturers to consider and study both enantiomers when selling a racemic mixture, or to commercialize only the eutomer [17,18]. However, it requires to either produce only the eutomer (need for enantioselective chemical methods) or to produce racemic mixture and resolve it (that is most of the time expensive and generates waste). Getting enantiomerically pure drugs is still a tough task as 88 % of current chiral drugs are sold as racemic mixture (thus, the distomer has also been clinically tested) that is often a cheaper solution [10].

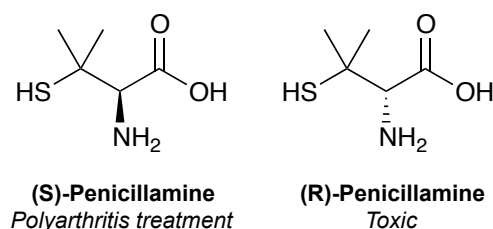


Figure I - 2 – Penicillamine enantiomers. The enantiomers of Penicillamine have different properties: the S-eutomer is a drug used in the polyarthritis treatment; the R-distomer is toxic.

2.1.1 Chiral synthesis by chemo-catalytic pathways

There are globally two ways to produce chiral compounds using classical chemistry: the asymmetric synthesis and the chiral resolution [2].

In the first approach (asymmetric synthesis), only one enantiomer is produced, leading to a maximal theoretical yield of 100 %, starting from a non-chiral reactant, or optically pure chiral reactant. There are three main kinds of asymmetric synthesis [10] : (i) an enantioselective catalyst aims at the production of chiral product. Typically, homogeneous organometallic catalysts are used [19]. They often require the use of toxic and polluting solvents, need high-energy inputs (e.g. high hydrogen pressure and high temperatures), and at the end of the process, the removal of the catalyst from the final product imposes further purification steps [20,21]. (ii) A chiral auxiliary is temporarily added to a compound to guide the reaction towards the production of desired enantiomer, and is then removed; (iii) the enantioselective compound is built starting from a chiral precursor (for example, an amino-acid) [22].

Practical examples of asymmetric synthesis are numerous in the pharmaceutical sector. Hereafter, we briefly present three examples for chiral amines. S-Clopidogrel (Figure I - 3) is the second world most sold medicine [3]. Its current production involves the reduction of a carbonyl centre into a chiral alcohol. This step is catalysed by ruthenium-based organometallic catalyst, $[\text{Ru}(p\text{-cymene})\text{Cl}_2]_2$, using a ligand to bring enantioselectivity to the process. Reaction is proceeded at 0 °C, requiring extra energy for cooling. Final product exhibits an enantiomeric excess of 92 % [23,24].

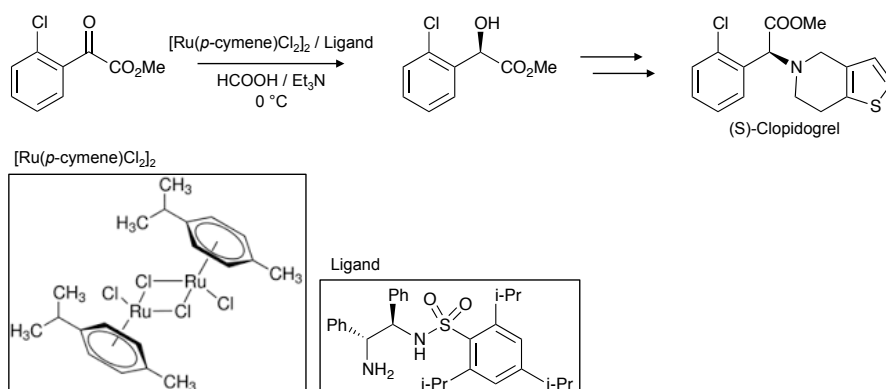


Figure I - 3 – S-Clopidogrel synthesis.

R-Sitagliptine (Figure I - 4) is a drug, produced by Merck, and used in the treatment of type II diabetes. Its chemocatalytic synthesis, that was implemented for decades, involved the use of an organometallic enantioselective catalyst ($\text{RuCl}_2 \cdot \text{P(Ph)}_3 \cdot \text{JOSIPHOS}$) containing a phosphine group. After the imination of a ketone group, the imine group is reduced under high-pressure hydrogen (17 bars) with the enantioselective catalyst. Final enantiomeric excess reaches 97 % [25]. Recently, the biocatalytic pathway involving transaminases (*vide infra*) has been implemented by Merck.

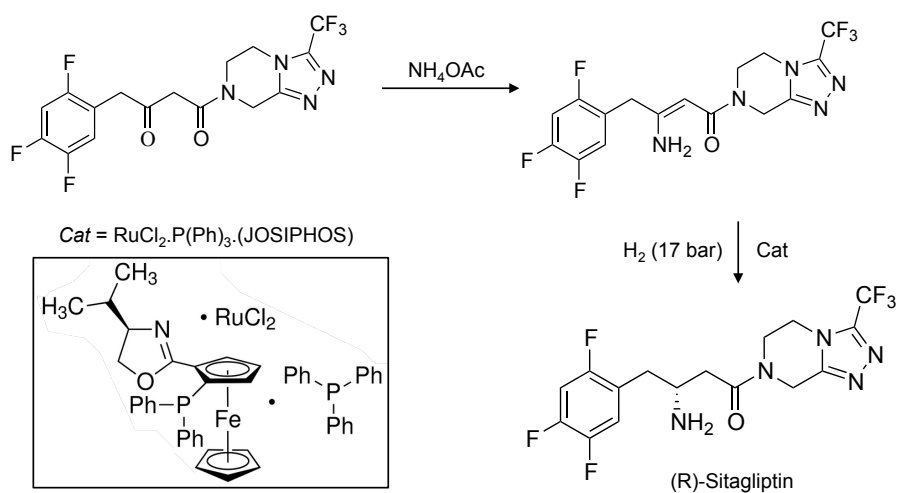


Figure I - 4 – R-Sitagliptine synthesis.

S-Rivastigmine (Figure I - 5) is a chiral amine used in the treatment of Alzheimer disease. The synthesis pathway involved the enantioselective transformation of a ketone into a chiral alcohol. This

step is catalysed by iridium-based enantioselective catalyst, under gaseous hydrogen (10 atm). Further steps, leading to the S-Rivastigmine involve change in temperature (from 50 to 0°C, then 90 °C) and toxic organic solvent (tetrahydrofuran) [26]. Recently, the biocatalytic pathway has been implemented (see Section 2.2.5).

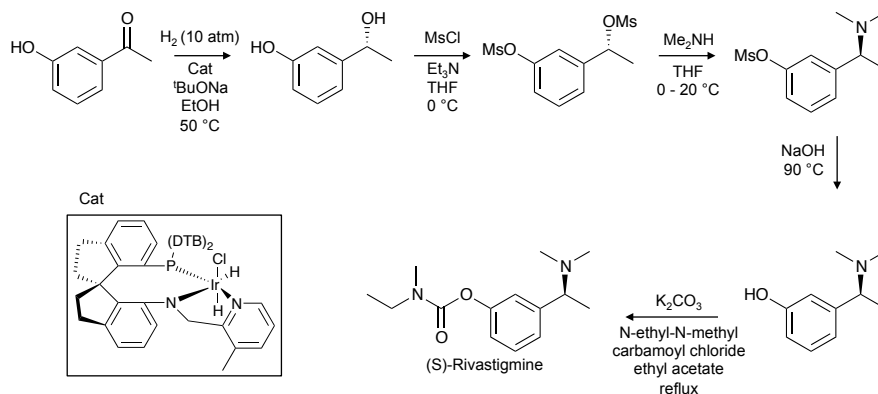


Figure I - 5 – S-Rivastigmine synthesis. MsCl stands for methanesulfonyl chloride.

In the second approach (chiral resolution), a racemic mixture is first produced. Then, the enantiomers are separated and only the eutomer is kept while the distomer has to be eliminated (or recycled in the most favourable cases). Thus, the maximal theoretical yield of the chiral resolution is only 50 %, as half of the product is not used. This non-green method is applied in the industry when no suitable enantioselective catalyst can be found to perform an asymmetric synthesis [27]. Preparative chiral High Performance Liquid Chromatography (HPLC) – based on the use of chiral stationary phase – can in some cases be used to separate enantiomers [28]. However, most methods for enantiomers separation are based on the formation of diastereoisomers (enantiomers that are combined with optically pure chiral compound) [10]. Diastereoisomers can then be separated according to their physico-chemical properties. For example, when a mixture of diastereoisomers is cooled, one compound crystallizes first and can therefore be removed from the medium while the other compound remains in solution. A liquid mixture of diastereoisomers can also be separated by preparative HPLC on a non-chiral phase. Those methods are conducted at high costs (solvents, duration, waste of reactants).

To illustrate the chiral resolution approach for the synthesis of a chiral amine, Figure I - 6 shows the synthesis of (S,S)-Sertraline. A S-

imine precursor is produced first, starting from the ketone and using methylamine as a reagent in hot toluene. Then the non-enantioselective reduction of the imine is carried out under pressurized hydrogen with a palladium reduction catalyst. Thus the intermediate product is a diastereoisomeric mixture of (S,R) and (S,S) compounds. The last step consists in the separation of diastereoisomers, using R-mandelic acid: the diastereoisomers (S,S)-Sertraline forms an insoluble salt with R-mandelic acid, that precipitates and is easy to recover. The other salt (S,R)-Sertraline-mandelate stays soluble in the solution [29]. The maximal yield is only 50 % [30,31].

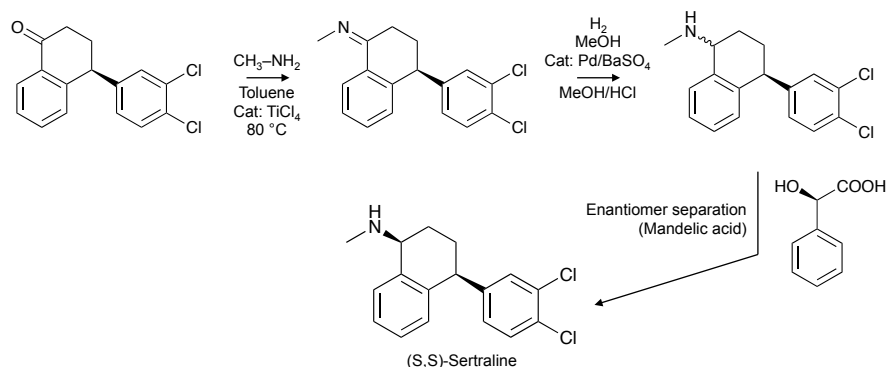


Figure I - 6 – (S,S)-Sertraline synthesis. The eutomer precipitates with R-mandelic acid while the distomer remains soluble, therefore allowing chiral separation.

Another strategy is the kinetic resolution: starting from a racemic mixture, an enantioselective catalyst aims at the consumption of the distomer, while the eutomer stays unreacted. This method of resolution, that needs an efficient enantioselective catalyst, is of particular interest when the direct asymmetric synthesis of the eutomer is not thermodynamically favoured. Thus, for such reactions, starting from a racemic mixture, the distomer is removed from the reaction medium as it is converted in product, keeping the eutomer unreacted.

All in all, the common routes to chiral compounds production following the chemocatalytic methods often do not respect the green chemistry principles. Indeed, those synthesis methods use toxic catalysts (that could remain in the final product), reach incomplete enantioselectivity (requiring further purification and separation steps), involve massive waste production (owing to the multi-step approach), and use organic solvent (potentially toxic and polluting). Toxic or

dangerous reactants are often used (e.g. pressurized hydrogen, methylamine, alkaline compounds). Such syntheses induce high-energy consumption (heating, cooling steps, pressurized gas).

2.1.2 Chiral synthesis by biocatalytic pathways

Enzymes are the catalysts used by living organisms. Enzymes are made of protein chains. They usually exhibit high activity: most of them show turnover rate between 10 and 10000 catalytic acts per second. They are also highly (often perfectly) enantioselective. Enzymes usually work in mild conditions: aqueous media, mild pH, and low temperature. Cherry on the cake: they are perfectly biodegradable and non-toxic (traces of enzymes in the final product would not represent an important issue in many cases) [32].

Various characterized enzymes are currently available in large library, ready for industrial use [33]. Moreover, thanks to the great advances in genetic technologies of the last decades, it is now possible to engineer enzymes to obtain mutated variants endowed with desired properties, e.g. higher resistance to organic solvent, higher temperature or pressure resistance, tuned (enantio/regio)-selectivity, larger substrate specificity [7,34]. Thus, enzymes seem to be now the ideal candidates to process chemical reactions of industrial interest in a greener way. Eventually, they can first be used to replace some steps in a given chemical process, thus in combination with non-enzymatic steps [35,36].

In the case of chiral amines, many enzymes are able to catalyse reactions leading to the production of compounds of interest. A selection of these is presented in the Figure I - 7 and Figure I - 8. Lipases have been used by BASF to produce chiral amine from racemic mixture, by catalysing enantioselective amidation. Then, at the end of the reaction, the reaction mixture contains optically pure chiral amines and optically pure chiral amides, with excellent enantioselectivity (Figure I - 7a) [37].

L-selective amidases have been implemented by DSM to produce non-proteinogenic amino acids (*i.e.* that are not naturally found in living organisms): the reaction catalysed the conversion of a racemic mixture of 2-aminoamides, into optically pure L-amino acids and optically pure D-amino amides (non-proteinogenic; Figure I - 7b) [37].

Similarly, hydrolases catalyse the enantioselective hydrolysis of amides into chiral amines starting from a racemic mixture of amides (Figure I - 7c) [2].

Ammonia-lyases (AL) are a class of enantioselective enzymes that are able to add ammonia on a carbon double bond, leading to chiral amines. Among them, aspartic acid ammonia-lyase (AAAL) has been used for the industrial production of Aspartame (sweetener) by Holland Sweetener Company: the addition of ammonia on fumaric acid led to L-aspartic acid, precursor of Aspartame (Figure I - 7d) [37].

Tyrosine Ammonia-lyase (TAL) catalyses the enantioselective addition of ammonia on coumaric acid, giving L-tyrosine amino acid (Figure I - 7e) [38]. The use of TAL for enantioselective transformations is the subject of the Chapter VI.

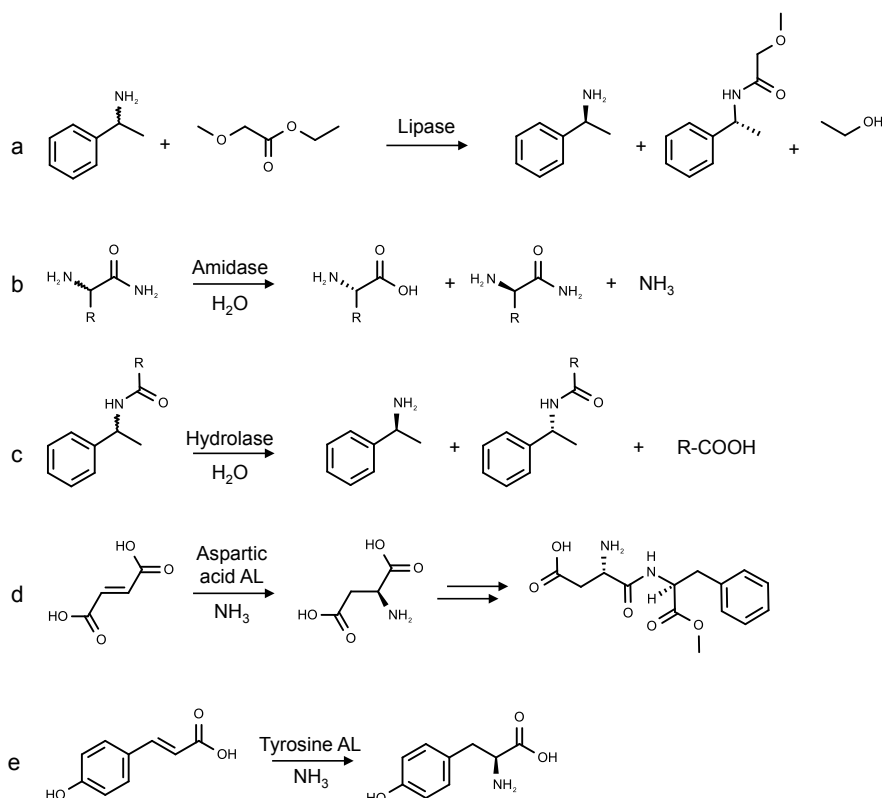


Figure I - 7 – Selection of enzymes catalysing chiral amines production. (a) lipase, (b) amidase, (c) hydrolase, (d) aspartic acid ammonia-lyase (AAAL) and (e) tyrosine ammonia-lyase (TAL).

Chiral amines can be produced starting from imine compounds. The reduction of imine functions is catalysed by imine reductases (Figure I - 8f). However, this process is limited by the low amount of

substrates that are accepted by the enzyme (mostly cyclic secondary imines) [2,39].

P450 monooxygenase (Figure I - 8g) is an enzyme that catalyses the chiral intra-molecular C-H amidation, leading to cyclic chiral amines [40].

Amine dehydrogenases and amino-acid dehydrogenases (Figure I - 8h) catalyse the reductive amination of carbonyl centres into chiral amines. Such enzymes catalyse the addition of ammonia on carbonyl centre, using NADH as a cofactor [39,41].

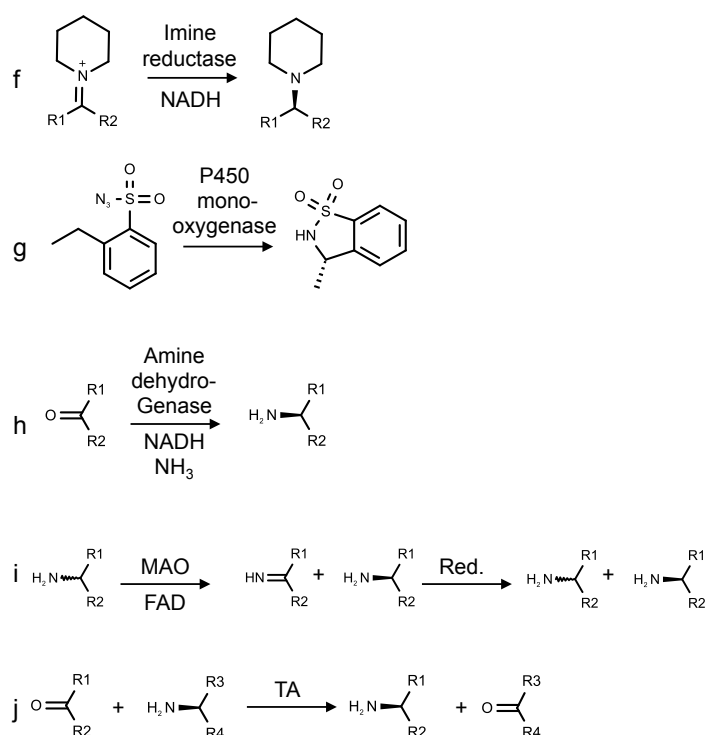


Figure I - 8 – Selection of enzymes catalysing chiral amines production. (f) Imine reductases, (g) P450 monooxygenase, (h) amine dehydrogenase, (i) monoamine oxidase (MAO); and (j) transaminase (TA). NADH stands for nicotinamide adenine dinucleotide reduced form, and is a reductant. FAD stands for Flavine Adenine Dinucleotide

Monoamine oxidases (MAO) catalyse the enantioselective oxidation of C-N bond into C=N, using FAD as cofactor (flavine adenine dinucleotide; Figure I - 8i). Large libraries of variants of various selectivities have been produced. The chiral amine of interest is obtained by deracemization of racemic mixture, in combination with

chemical reducing agent (leading to the accumulation of the desired enantiomer) [42].

Finally, transaminases (TA), also called amino-transferases, are enzymes that catalyse the enantioselective transfer of an amino group from an amine (amino-donor) to a ketone (amino-acceptor; Figure I - 8j) [2]. Transaminases are the focus of the present thesis and will be intensively discussed in the next section.

2.2 Transaminases

2.2.1 Classification

For the transamination catalytic act, transaminases need a cofactor, namely pyridoxal 5'-phosphate (PLP, Figure I - 9) [43]. This molecule is biosynthesized from vitamin B6. Around 240 different reactions are catalysed by PLP-dependent enzymes that are divided into seven classes (based on the enzymes structures): (I) aspartate transaminase, (II) tryptophan synthase, (III) alanine racemase, (IV) D-alanine transaminase, (V) glycogen phosphorylase, (VI) D-lysine-5,6-aminomutase and (VII) lysine-2,3-aminomutase. PLP-dependent enzymes catalyse numerous reactions: racemization, transamination, decarboxylation, elimination, retro-aldol cleavage, Claisen condensation; always involving the PLP as a cofactor. Some of those enzymes are also involved into the amino-compounds metabolism (involving amino acids, amino-sugars, polyamines, etc.) [8,43].

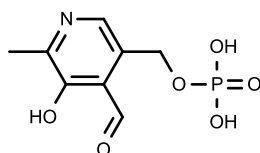


Figure I - 9 – Pyridoxal 5'-phosphate (PLP), the cofactor of PLP-dependent enzymes.

Among those enzymes, transaminases (TA) are PLP-dependent enzymes of types I and IV (depending on the folding). In living organisms, TA are involved in the synthesis and the degradation of amino acids, and in the synthesis of secondary metabolites [44–46].

Transaminases catalyse the enantioselective and reversible transfer of a primary amino function, from an amino-donor, to an amino-acceptor (comprising a carbonyl function, see Figure I - 8j). Specifically, α -TA catalyse the transfer of amino function between

alpha-amino acids and alpha-keto acids (the acceptor-carbonyl functions is directly bound to a carboxylic function). ω -TA catalyse the amino transfer on a non-alpha carbon (in addition to the acceptor-carbonyl function, the acceptor molecule may contain a carboxylic function, but is not located on the α -carbon). Among ω -TA, the amine-transaminases, ATA, are enzymes that catalyse the amino transfer on amino-acceptor, no matter if it contains a carboxylic function in addition to the carbonyl-acceptor function. Thus, ATA enzymes can catalyse the transamination of ketone and aldehyde compounds [8,47,48].

Among ATA transaminases, one has been intensively characterized [49,50]: the R-selective ATA transaminase from the soil microorganism *Arthrobacter* sp. KNK 168. A homologue of this enzyme (99.7% identity) has been commercialized by Codexis, as ATA-117, and the 3D structure has been recently published [51]. This last enzyme, ATA-117, is the transaminase mainly used in this work. Indeed, this enzyme has been intensively studied on the last decade. It has been the starting point for the production of an engineered enzyme, ATA-117-11Rd, that successfully catalyse the production of R-Sitagliptin [7], and Suvorevant [52,53]. ATA-117 (the non engineered version) is commercialized and easily purchasable, making it an ideal candidate for our work.

2.2.2 ATA-117 Structure, specificity and mechanism

ATA transaminases are homo-dimeric enzymes. Two half-active sites are located on each monomer. The two monomers must be assembled for the active sites to be catalytically active [54] (Figure I - 10, left).

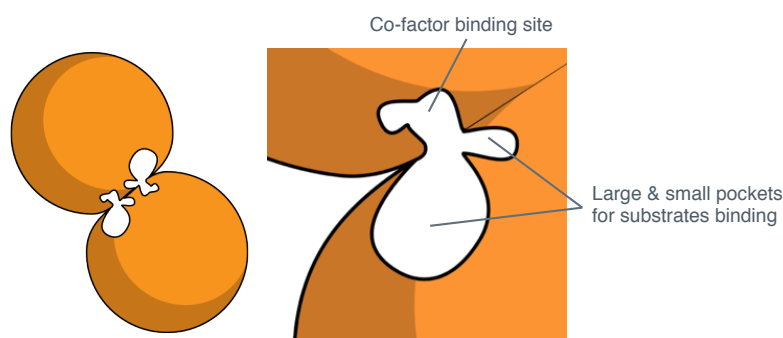


Figure I - 10 – Schematic representation of ATA enzymes. (Left) Scheme of ATA homo-dimers. Subunits must be assembled to form two active sites that are catalytically active. Two active sites are located at the interface between subunits. (Right) Schematic representation of an active site.

The active site is made of three pockets: one is dedicated to the PLP binding (Figure I - 10, right), the two others are assigned to the substrate binding. The PLP-binding pocket exhibits an essential lysine residue for the covalent reversible fixation of the cofactor (see mechanism, Figure I - 11). The small substrate pocket only accepts small organic functions (e.g. methyl groups). The large pocket is more versatile and accepts larger organic groups (alkyls chains, carboxylic acids, ethers functions, aromatics, etc.) [34]. Thus, ATA-117 is able to catalyse the formation/consumption of amines that contain a methyl group on one side, or smaller substituents, and larger organic functions on the other side of the amine.

Transaminases follow a “ping-pong bi-bi” mechanism [8,55,56] (Figure I - 11): the enzyme catalyses two successive bi-molecular reactions (hence “bi-bi”), the second one is the exact reversed sequence of the forward one. The enzyme reaches an intermediate state after the first half-reaction, and “bounces back” into its initial state after the end of the second half-reaction (hence “ping-pong”).

The first step (Figure I - 11a) consists in the formation of internal aldimine: the cofactor is covalently, but reversibly, bound to the lysine residue located in the PLP-binding pocket. Then, the amino-donor (here, represented as an amino acid, e.g. D-alanine) enters into the active site and is placed in its binding pocket. The shape and the physicochemical properties of the active site force the amine to bind in a unique orientation, hence the enantiospecificity of the transaminase [44]. Then, by imine-amine exchange, the amine function (from the amino-donor) is substituted to the lysine residue (bounded to PLP), and binds the cofactor in a transamination reaction (the lysine anchoring point is then released). In the next step (Figure I - 11c), a basic residue located in the active site tears off a proton located on the alpha-carbon and the pyridinium ring of the cofactor pulls the delocalized electrons. Electrons are then pushed back and the cofactor is protonated on the other side of the substrate nitrogen by acid catalysis. Hence, electron displacement and acid/base catalysis induce the formation of a so-called external imine *i.e* where the imine is on the other side of the nitrogen. This external imine is maintained in the active site due to electrostatic and hydrophobic interactions. The last step of the first half-reaction (Figure I - 11d) consists in the hydrolysis of the imine, releasing a carbonyl compound as a by-product (here, an α -keto acid). The cofactor stays in the active site in an aminated form, namely pyridoxamine (PMP). Thus, the first half-reaction consists in the deamination of the amino-donor (transformed into a keto derivative) and the amination of the PLP (into PMP). Then, the second half-reaction occurs in the exact

reverse sequence of events (Figure I - 11 from e to h), but using an amino-acceptor and releasing a chiral amine [57–59].

As the transamination reaction is an equilibrated chemical reaction, this mechanism can be read in both ways.

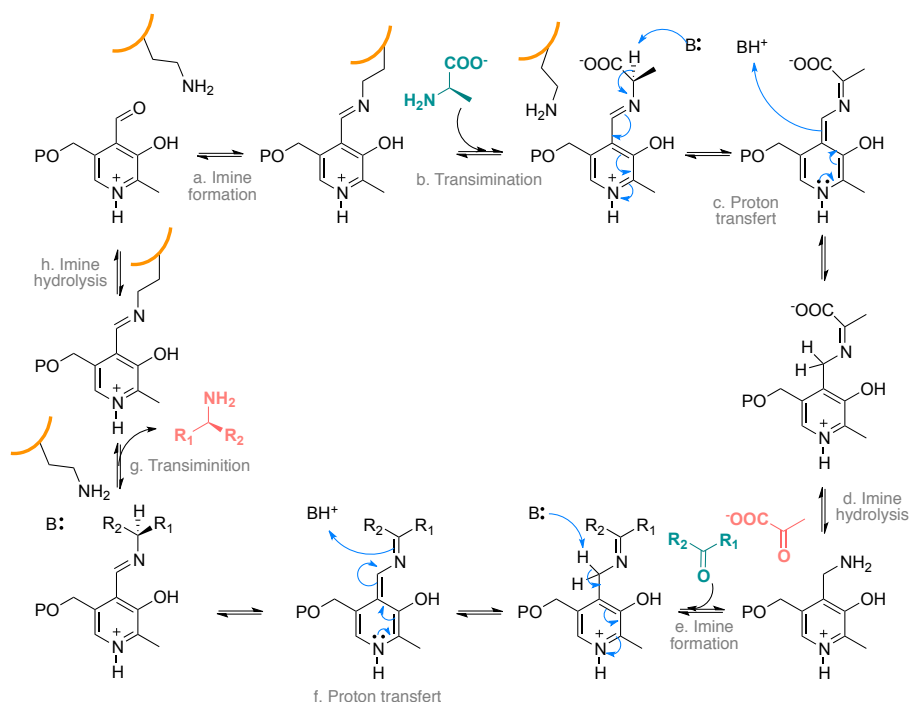


Figure I - 11 – Transamination mechanism. Two half-reactions execute the ping-pong bi-bi mechanism: from (a) to (d), the amine reacts with the PLP cofactor to form the ketone by-product and a PLP amine-derivative (PMP, Pyridoxamine phosphate); from (e) to (h), the ketone reacts with the PMP to form the amine and release the PLP. PO stands for phosphate, and B for basic molecule or residue. Adapted from [57–59].

From an industrial perspective, and moreover when working with immobilized enzymes (see Section 2.3), it is relevant to know the structure and the surface composition of the enzymes. Indeed, knowing about the enzyme surface helps to choose the optimal immobilization method. The dispersion of amino-acid residues on the enzyme surface can also impact the activity of the immobilized enzyme (e.g. through orientation effects).

The three-dimensional structure of ATA-117 has been published [51], and a PDB (for Protein Data Base) entry was assigned (and is 3wwh). Using the PISA tool [60] (for Proteins, Interfaces and

Structures Assemblies), it is now possible to know the relative position of each amino acid: (i) solvent-accessible residues (external surface), (ii) interfacing residues, or (iii) inaccessible residues (located into the enzyme structure). Figure I - 12 shows the chemical composition of amino acids located at the external surface (solvent-accessible residues).



Figure I - 12 – ATA-117 Protein sequence, structure and surface composition. (a) ATA-117 protein sequence. Underlined residues are located at the external surface of the enzyme. Colours refer to chemical types as defined in (c). (b) ATA-117 monomer unit (modelization based on protein sequence) (c) Ratios of chemical functions at the external surface: more than half of the amino acids located at the surface are hydrophobic residues. A large part of the residues are polar or charged.

Each monomer of ATA-117 exhibits 3 aminated residues (lysine, K) at its surface. Amino-terminal residue (that contains an amino-moiety) is not exposed to the surface. A large part of surface residue are charged or polarized. The monomer surface is mainly hydrophobic (~ 60 % of surface amino acids). A large part of such hydrophobic residues are located at the interface between subunits. Isoelectric point of ATA-117 was estimated at 4.7 (prediction from sequence, using ExPASy Tool [61–63]).

2.2.3 TA catalysed reactions and limitations

Chiral amine can be produced along with three different enzymatic reactions using ω -transaminases [48,64] (Figure I - 13): (i) the kinetic resolution, (ii) the asymmetric synthesis, and (iii) the deracemization.

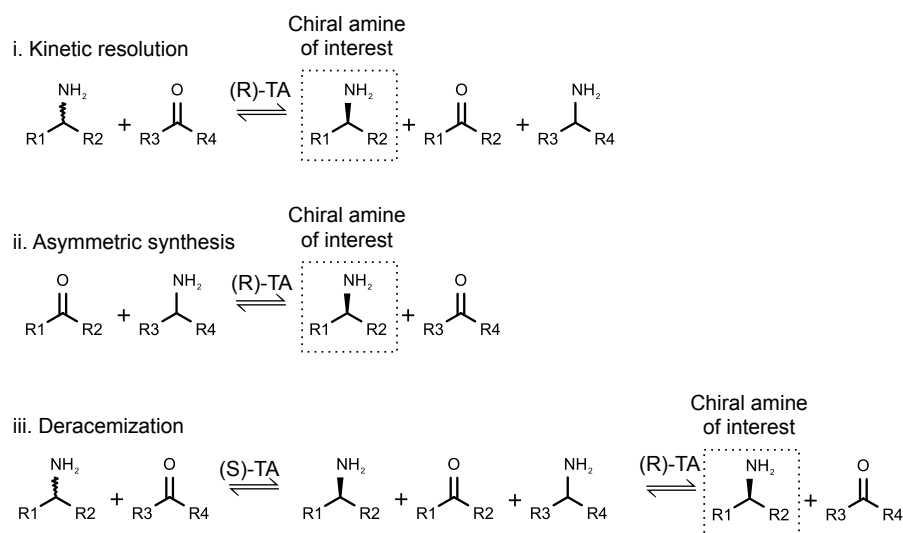


Figure I - 13 – Reactions catalysed by transaminases for the production of chiral amines.

The kinetic resolution: starting from a racemic mixture of enantiomers, an enantioselective enzyme converts only one enantiomer of the initial mixture into the corresponding keto-compound. At the end of the kinetic resolution, one half of the racemic mixture has been converted into ketone, the other half, enantiomerically pure, stays unconverted. An aminated by-product is also present in the final mixture. Thus, the maximal yield for the production of chiral amines using the kinetic resolution is only 50 %. Also, when using amines (not amino acids) as amino donor, ketone product is accumulated in the reaction medium although ketones are known to inhibit transaminases (unlike keto acids that are produced when using amino acid as amino donor, like in the biological systems) [44,48,65]. The main advantage of the kinetic resolution is that it only requires only one enantioselective enzyme [57].

The asymmetric synthesis: a pro-chiral ketone is converted into a chiral amine, using an amino-donor, and producing a ketone by-product. If the enzyme is perfectly enantioselective and the chemical

equilibrium position is favourable, the maximal yield is 100 % as all the pro-chiral ketone is transformed into chiral compound. Moreover, this method only requires one enzyme. However, the asymmetric synthesis suffers from two severe limitations: first, most transaminases are inhibited by ketone compounds (the by-product, that is produced if amino donor is an amine – e.g. isopropylamine). Second, the asymmetric synthesis of chiral amines (that are not amino acid) is not favoured thermodynamically [48,57]. In fact, in the Nature, transaminations between α -keto acids and α -amino acids are fairly equilibrated reactions, because the ability of α -keto acids to be aminated is close to the ability of amino acids to be deaminated [44]. However, thermodynamically, amines (*i.e.* not amino acids) are easily deaminated (as compared to amino acids), while ketones (as compared to α -keto acids) are hardly aminated (Figure I - 14). Thus, the chemical equilibrium is driven towards the amine deamination [44].

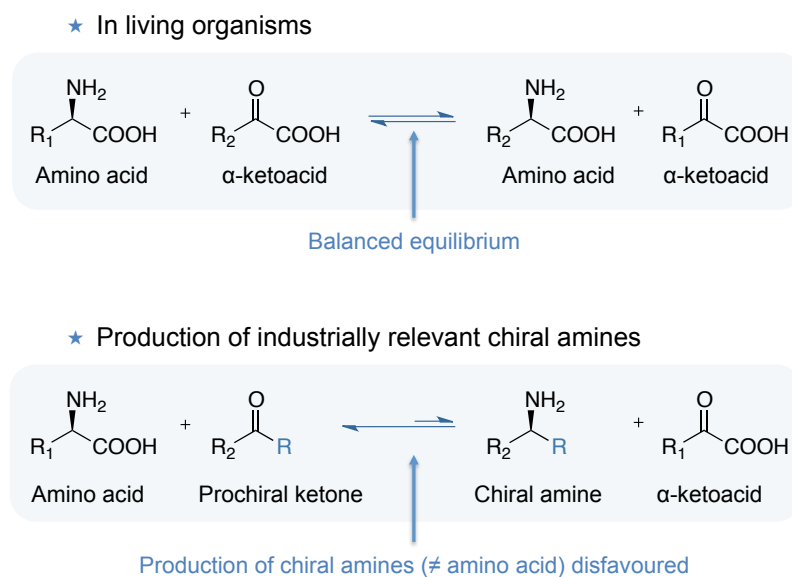


Figure I - 14 – Transamination for the production of chiral amines. Transaminations that involve amino acid and α -keto acids (as mostly the case in living organisms) are governed by balanced equilibrium. As the chemical and pharmaceutical industries need to produce chiral amines (that are not amino acids, *i.e.* R is not a carboxylic acid), the transamination requires the use of ketones. However, ketones are not easily aminated. Thus, the equilibrium reaction for the production of chiral amines is not favoured.

Deracemization involves a kinetic resolution, starting with a racemic mixture, followed by an asymmetric synthesis. First, an enantioselective transaminase (*e.g.* S-selective TA) converts an

enantiomer from the racemic mix into the corresponding ketone. Then, a second transaminase of reversed enantioselectivity (e.g. R selective TA) catalyses the asymmetric synthesis of R product, using the ketone formed during the kinetic resolution step. Thus, by this method, the maximal theoretical yield is 100 %, and no inhibiting ketone is accumulated, as only α -keto acid is accumulated at the end of deracemization (if starting from α -keto acid as amino acceptor). However, this method requires two enzymes (and suffers from unfavourable thermodynamics in the asymmetric synthesis step) [66].

Thus, the use of transaminases for the production of chiral amines suffers from two major limitations: (i) when using amines as amino donor, the transaminases are strongly inhibited by the ketone by-product; and (ii) the thermodynamics does not favour the production of chiral amines (that are not amino acids). Those limitations do not allow the enzymatic production of chiral amines at industrial scale (*i.e.* high yield and high product concentration). Therefore, strategies have been developed to obtain chiral amines at high yield, and high concentration (*vide infra*).

2.2.4 Enhancement strategies

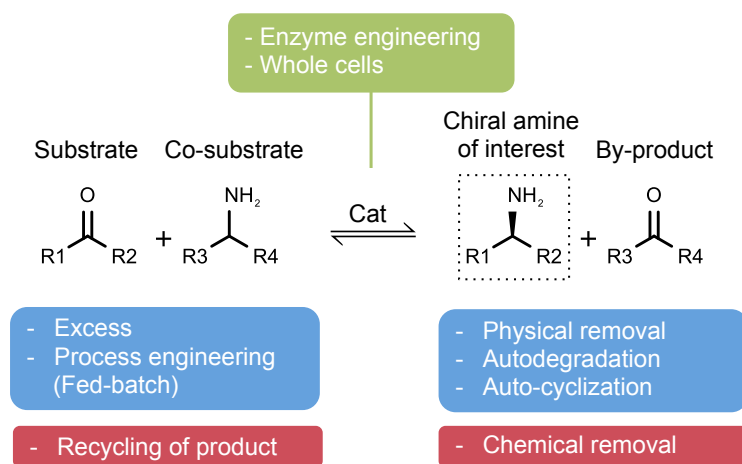


Figure I - 15 – Enhancement strategies. Strategies for fighting against ketone inhibition and unfavourable thermodynamics.

Strategies to enhance the chiral amines production by transaminases have been developed (to counter enzyme inhibition by ketones and unfavourable thermodynamics). They can be split in three categories (Figure I - 15): (i) the strategies aiming at enhancing the enzyme performance or robustness, (ii) strategies aiming at shifting the equilibrium by physico-chemical effects (mono-enzymatic

methods), and (iii) strategies involving several enzymes in addition to TA (multi-enzymatic methods) [44,67]. In practice, many studies have combined several strategies [68], and the classification is not so straightforward.

2.2.4.1 Strategies aiming at enhancing the enzyme performance or robustness

At the enzyme scale, there are two approaches to prevent enzyme inhibition by ketone compounds: the enzyme engineering and the use of whole cells.

The enzyme engineering strategy allows the production of enzyme mutants that exhibit new properties as compared to the wild type enzyme. For example, engineered transaminase could be more tolerant to high substrate and/or product concentrations. The resulting enzyme would not be inhibited anymore (or at a lower level) [44].

This strategy allows to get enzymes that are more suitable for industrial applications: stability under non physiological conditions (high concentrations, organic solvent), working and storage duration [44]. In practice, there are two ways to produce mutated enzymes [48].

First, a random mutagenesis approach associated with a screening can be envisioned. In this strategy, the enzyme structure is not necessarily known. Random mutations are introduced in the gene of interest (encoding the enzyme). A library of microorganisms expressing the enzyme variants is then created. Then, the library is screened to identify the colonies that express the enzyme exhibiting the interesting properties. The interesting colonies are then selected. For example, Yun *et al.* [69] created and identified a mutated version of *Vibrio fluvialis* transaminase that shows better tolerance to aliphatic ketones (as compared to the wild-type transaminase).

The second method for mutated enzymes production is based on site-specific mutagenesis. This requires knowing the enzyme structure. By a rational design approach (identifying the link between amino acid sequence and enzyme 3D structure), it is possible to predict the properties of a desired mutant where a specific amino acid has been replaced by another (predictions and modelization *in silico*) [48]. For example, Savile *et al.* [7] used a substrate walking approach to predict the effects of mutations of the active site on the substrate pocket affinity. The wild type enzyme (ATA-117) did not accept large substrate in its pocket. After 11 rounds of mutations (a combination of

site-directed and random mutations), a mutated enzyme (ATA-117-11Rd, exhibiting 27 mutations) was able to accept larger substrate in its active site pocket. Then, the mutated enzyme was able to catalyse the synthesis of Sitagliptine, an anti-diabetic drug (see Figure I - 1), with excellent yield and enantioselectivity. Moreover, the mutated enzyme exhibits better tolerance to organic solvent (DMSO) and amino-donor (isopropylamine). The major disadvantage of enzymatic engineering, however, is the high cost of mutated enzyme production [44].

Another strategy consists in the use of whole cells to catalyse the transamination reaction. In this case, the interesting transaminase is overexpressed in living cells, which are then used as such as living catalysts. Thus, the enzymes are protected towards extreme environment conditions by the natural cytoplasmic membrane. For example, Koszelewski *et al.* [70] were using *Escherichia coli* cells (containing an over-expressed transaminase) to produce aliphatic chiral amines by kinetic resolution. The main disadvantages in the use of whole cells are (i) substrates and products must be able to penetrate and come out of the cells ; or enzyme must be secreted; (ii) the need to feed the living cells, and the potential interferences with other metabolic pathways, that could alter the purity of the final product [71].

2.2.4.2 Mono-enzymatic methods

In the mono-enzymatic methods, only one catalyst is involved: the transaminase that catalyses the transamination. The equilibrium is shifted either by using an excess of substrate, or by removing a product. Also, process engineering can control the inhibition.

An excess of isopropylamine (50-fold excess) has been successfully used by Truppo *et al.* [72] in the amination of acetophenone, using ATA-117. The product (methylbenzylamine) was obtained at high yield (95 % conversion) and high enantiomeric excess (> 99%).

Inhibition caused by the substrate (in high concentration) can be avoided by process engineering: a fed-batch strategy allows feeding fractionally the reaction medium with the substrate, so that the concentration is always controlled and is never too high to inhibit the transaminase. Rios-Solis *et al.* [73] used the fed-batch strategy for the progressive addition of isopropylamine in their bioreactor (containing a transketolase and a transaminase). They synthesized amino-alcohol product with satisfying yield (70 %), avoiding the

inhibition caused by high isopropylamine concentration (due to alkaline properties of isopropylamine).

Another strategy used to shift the equilibrium towards the asymmetric synthesis consists in the removal of products and by-products [44]. Park *et al.* [74] use isopropylamine as amino-donor for the production of L-homoalanine, leading to the co-production of acetone that is easily eliminated from the reaction medium by evaporation. Products and co-products can also be extracted from the reaction medium by liquid-liquid or liquid-solid extractions [75–78].

Products can also be removed from the system by an auto-conversion pathway. Green *et al.* [79] propose the use of ortho-xylylenediamine as amino-donor for the transamination (using ATA-256). The by-product (1H-isindole) spontaneously undergoes tautomerization, leading to the formation of 2H-isindole that polymerizes and precipitates into a blue compound. This spontaneous product tautomerization and subsequent precipitation drives the chemical equilibrium towards the production of S-2-formylbenzylamine.

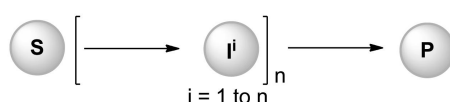
Products removal has also been conducted through co-product cyclization. For example, by using lysine as amino-donor, Lo *et al.* [80] produced L-homophenylalanine at high yield (97 %) and high enantiomeric excess (>99.9 %) as the by-product spontaneously cyclizes and then, drives the equilibrium. Also, this cyclization allows avoiding the inhibition by the by-products.

2.2.4.3 Multi-enzymatic methods

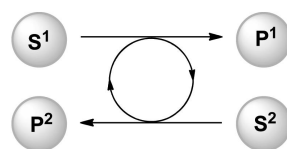
Multi-enzymatic methods involve more than one enzyme for the asymmetric synthesis. Other enzymes allow either consuming the by-product, or recycling the by-product into substrate. By using enzymes purified from similar cell media, multi-enzymatic methods (also called enzymatic “cascades”) are easily practicable in one-pot system [34]. There are four kinds of enzymatic cascades (Figure I - 16). (a) In the linear cascade, the substrate is converted into the product, through the intermediate successive steps that lead to the formation and consumption of intermediate compounds. Intermediate compounds do not need to be isolated. This is convenient in the case if the intermediate compound is hazardous to handle. (b) In the parallel cascade reactions, two reactions are occurring simultaneously. The reactions share some substrate and product. They are widespread in the metabolic pathways: one reaction is catalysed by an enzyme that needs a cofactor (e.g. NADH) that is transformed (e.g. oxidized

into NAD^+). The second reaction is catalysed by an enzyme that uses the transformed form of the same cofactor, and regenerates it. In an applied perspective, these convenient cascades allow the recycling of expensive cofactors. (c) In the orthogonal cascade, a first reaction allows the production of product of interest and a by-product (or intermediate) that is transformed in a second reaction. This kind of cascade allows the equilibrium displacement (by withdrawing the by-product) towards the production of product of interest (P_1 on the picture). (d) In the cyclic cascades, one product is converted back to a substrate. This strategy is used for stereo-inversion. For example, the kinetic resolution reaction is a kind of cyclic cascade where one enantiomer is produced, and accumulated in the reaction medium.

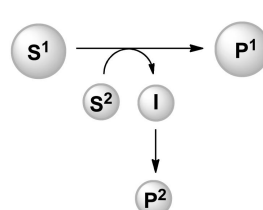
a.) linear cascade



b.) parallel cascade



c.) orthogonal cascade



d.) cyclic cascade

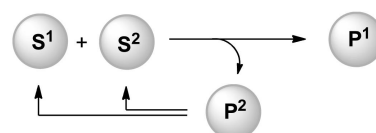


Figure I - 16 – Overview of different types of enzymatic cascades. S, I and P respectively stand for starting material, intermediate, and product. Figure and caption taken from [34].

Concerning the methods for favouring the asymmetric synthesis, one strategy consists in the consumption of by-product. When alanine is used as amino-donor, the transamination led to the production of pyruvic acid (or pyruvate salt) as a by-product. Several strategies have been implemented to catalyse the consumption of pyruvate to shift the equilibrium (orthogonal cascade scheme).

For example, lactate dehydrogenase (LDH) allows the transformation of pyruvate into lactic acid, using NADH cofactor (Figure I - 17) [64,72,81,82]. This strategy has been successfully used by Meadows *et al.* [75] for the production of S-1-(5-

fluoropyrimidin-2-yl)ethylamine. Moreover, the amine of interest was extracted in an *in situ* product removal (ISPR) approach by supported liquid membrane (SLM), leading to 98 % yield, and high amine purity.

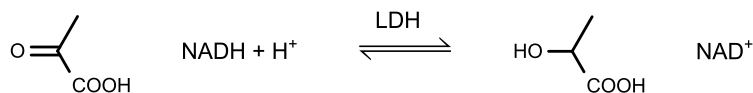


Figure I - 17 – Reaction catalysed by lactate dehydrogenase. Lactate dehydrogenase (LDH) allows to transform pyruvic acid into lactic acid, using NADH.

Other enzymes allow transforming pyruvic acid. For example, pyruvate decarboxylase catalyses the decarboxylation of pyruvate into acetaldehyde and CO₂ [83]; and alanine dehydrogenase allows the recycling of pyruvate into alanine (the amino-donor), using ammonia and NADH as a cofactor [84].

If isopropylamine is used as amino-donor, acetone is produced as by product. Then, alcohol dehydrogenases (e.g. yeast alcohol dehydrogenase, YADH) can catalyse the reduction of acetone into isopropyl-alcohol, as proposed by Cassimjee *et al.* [85] (Figure I - 18).

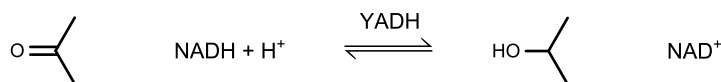


Figure I - 18 – Reaction catalysed by yeast alcohol dehydrogenase. Yeast alcohol dehydrogenase (YADH) catalyses the reduction of acetone in the presence of NADH.

Many of those enzymes are using NADH as reducing agent, in stoichiometric amount. However, this reagent is highly expensive and thus, needs to be recycled. Two main enzymes allow the recycling of NADH (towards the implementation of parallel cascades): the glucose dehydrogenase (GDH) and the formate dehydrogenase (FDH). GDH catalyses the oxidation of D-glucose by NAD⁺ (the oxidized form of NADH) into δ -gluconolactone (releasing NADH; Figure I - 19). This method has been extensively studied [66,72,84,86]. Formate dehydrogenase catalyses the oxidation of formic acid in the presence of NAD⁺, into CO₂ and allows the recycling of NADH [85,87].

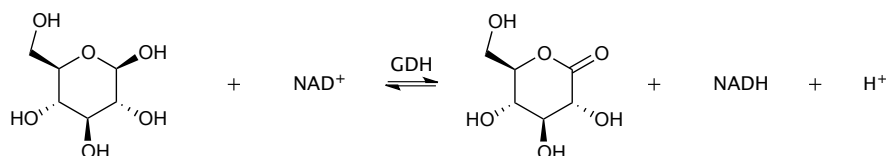


Figure I - 19 – Reaction catalysed by glucose dehydrogenase. Glucose dehydrogenase (GDH) catalyses the oxidation of glucose by NAD⁺ to form δ -gluconolactone and NADH.

2.2.5 Industrial applications

The use of transaminases allows shortening chemical synthesis pathways very significantly. Indeed, the implementation of transamination allows to avoid protection and de-protection steps, minimize waste generation, and leads to an overall higher atom efficiency [44].

Some transamination reactions are already developed at kilo- and industrial scales for drug manufacture e.g. the Sitagliptine (anti-diabetic drug), Suvorexant (sleep regulation) and Ivabradine (heart-rate regulation) production. R-Sitagliptine is produced by Merck at high concentration (250 g/L), in 50 % DMSO at 50 °C, using the engineered transaminase ATA-117-11Rd. The transamination reaction is implemented instead of a chemocatalytic step involving rhodium catalyst and high pressure hydrogen that was implemented for decades [7,8]. The same enzyme is used to produce Suvorexant, a sleep regulator: the transamination reaction replaces five chemocatalytic steps, leading to a key-intermediate. The amino-donor is methylbenzylamine. The overall yield reaches 40 % [8,52,53]. S-Ivabradine is synthesized using alanine, with a transaminase from Codexis. Excellent yield and enantioselectivity were obtained [8,88].

Finally, we must mention the production of S-methoxy-isopropylamine by an optimized transaminase from *Bacillus megaterium*, using isopropylamine as amino-donor. The product is a key-intermediate for the production of Metolachlor, a widely used herbicide [19].

2.3 Enzyme immobilization

2.3.1 Interest of immobilizing enzymes

The great efficiency of enzymes is well established (see Section 2.1.2). For the use of enzymes in industrial processes, the main drawbacks, however, are the lack of long term stability and the

difficulty in the recovering and recycling of enzymes [89,90]. Once the gene coding for the enzyme of interest is known, it is possible to produce enzymes at large scales (that allows to obtain them at low cost). However, some engineered enzymes encounter stability issues and need additional work (second round of engineering) for stability enhancement that lead to higher prices [91,92]. Considering their size, filtration is not straightforward (e.g. ATA-117 monomer measures 3 nm [51]).

Immobilizing the enzymes on larger carrier (*i.e.* solid support that efficiently maintains the enzymes) allows envisaging the facile recovery of the biocatalysts at the end of the reaction. And thus, it is possible to reuse the immobilized enzymes in further transformations. The facile separation of the solid biocatalyst from the reaction medium also allows minimizing or avoiding protein contamination of the product [93].

Furthermore, some immobilized enzymes have shown remarkable enhancement of their properties as compared to the free enzyme: e.g. higher activity [94], enhanced selectivity [95], improved stability towards storage and operational conditions [96,97]. For example, the lipase from *Pseudomonas fluorescens* (PFL) catalyses the enantioselective hydrolysis of (R,S)-2-hydroxy-4-phenyl-butanoic acid ethyl ester. The free PFL is poorly S-enantioselective (enantiomeric excess is 59 %) [94]. However, when immobilized on hydrophobic supports, a 7-fold increase in PFL activity is observed, and enantioselectivity is improved (≥ 92 %). Other outstanding examples are detailed in the further sections (and particularly, see Section 2.3.5 for properties of immobilized transaminases).

While most enzymes are inactive under organic conditions (as enzyme residues are not readily accessible to substrate molecules), immobilized enzymes often exhibit a higher activity in organic solvents (due to the enhancement of accessibility of enzyme active site and stabilization) [98]. Finally, immobilization allows the repeated use of the enzyme (catalyst recycling) or the implementation of continuous processes, where the enzyme is maintained in the reactor while the reaction medium is flowed through [89].

The main challenges of immobilizing enzymes are: (i) getting an efficient attachment of the enzymes on the carrier, to then avoid the enzyme leaching (*i.e.* when the enzyme detaches from the carrier); (ii) retaining the enzymatic activity when immobilized. In most cases, a compromise has to be found between a good immobilization and a good retention of enzyme activity [91].

2.3.2 Methods

Immobilization of enzyme has to be done case-by-case, as a function of the enzyme properties (surface composition, active site location), of the reaction of interest (operating conditions), of the solid carrier that is envisaged, and depending on the targeted improvements (stability, activity, substrate specificity, product selectivity, etc.). As there is no standard method, enzyme immobilization is mostly conducted through a trial-and-error approach [91,93].

Immobilization methods are generally sorted in three categories: (i) Enzyme hooked at the surface of a support, (ii) enzyme entrapment and encapsulation, (iii) enzyme cross-linking.

2.3.2.1 Enzyme attached at the surface of a support

This method of immobilization consists in the deposition of the enzyme on a ready-to-use carrier [93,99,100]. There are several kinds of immobilization of enzyme on a support: (i) simple adsorption, (ii) adsorption facilitated by electrostatic interactions, (iii) site-specific affinity attachment, (iv) adsorption facilitated by hydrophobic interactions, and (v) covalent grafting. Those methods have been implemented in this work (see Chapters I, III and IV).

The driving force of enzyme adsorption on a solid support has two components: an enthalpic part and an entropic part.

On the one hand, the enthalpic contribution consists in the electrostatic interactions and hydrogen bonds between the enzyme and the support. The adsorption would be facilitated for example if the protagonists exhibit opposite charges on their surface. A high ionic strength in the medium will however soften the electrostatic attraction [101]. Interactions with other enzymes (that are already immobilized) will also impact the potentiality of adsorption of a given enzyme: attraction between enzymes will favour the formation of a single enzyme monolayer, while a repulsive interaction will provoke the formation of a scanty enzyme layer. Thus those interactions will determine the density of enzyme layer [102]. Van der Waal interaction also reinforces the attraction between enzyme and support, and within the enzymatic layer [102].

On the other hand, the entropic contribution to adsorption driving force is related to the composition of enzyme surface. Enzymes are composed of amino acids, arranged in three-dimensional structure (helixes and sheets). Some amino acids are more hydrophobic, due

to their composition (aliphatic or aromatic carbon chains). In aqueous media, the enzyme structure is in the native conformation, *i.e.* the functional arrangement that minimizes the exposition of hydrophobic residues to the aqueous solvent. However, some hydrophobic residues are exposed to the solvent, and are then surrounded by highly ordered water molecule. As the enzyme is approaching the support surface, the highly ordered water molecules are desorbed, and the solid surface takes over to hide hydrophobic enzymatic surface. Thus, the expulsion of ordered water molecules contributes to the increase of entropy of the system, which is favoured thermodynamically [102,103]. When the support is a hydrophobic surface, this interaction is more favoured. However, if the hydrophobic interaction is too strong, it can lead to the denaturation of the protein (the 3D structure is modified to maximize the hydrophobic interaction, but is no longer able to do the catalytic act) [102,103]. The relative significance of both contributions depends on enzyme, support, and environmental conditions (pH, ionic strength, temperature) [102,104].

Thus, the immobilization can strongly affect the enzyme structure, and therefore, the enzymatic activity [96]. Moreover, if the interactions are located close to the active site, the last one can be hidden by the carrier surface, and in such case the enzyme can be unable to catalyse any reaction [105]. The link between the number of attachment sites and the enzyme stability has also been highlighted [106]: the more the enzyme is attached, the more the enzyme is stable in high thermal conditions.

- Simple adsorption

In this way of adsorption, the enzyme is simply deposited on the bare support surface. The immobilization occurs thanks to weak physical interactions (Van der Waals interactions, H bond, slight hydrophobic or electrostatic interactions). This method usually leads to important leaching (*i.e.* the enzyme is washed away from the carrier) and/or deactivation [93,107]. For example, the acrylic resin Amberlite XAD-7 was used to immobilize lipase (CALB) in a commercial biocatalyst, namely Novozym 435. However this catalyst is strongly deactivated in aqueous media, or in the presence of surfactant (that helps to leach the enzymes) [108]. A solution to the enzyme leaching consists in the deposition of a polydimethylsiloxane (PDMS) coating, that prevents the enzyme leaching but simultaneously allows the diffusion of reactants and products [93].

- Adsorption assisted by electrostatic interactions

This method is used when electrostatic interactions between the enzyme (which has a natively charged surface) and the charged support are strong enough to prevent enzyme leaching. In that way of immobilization, supports exhibit charged functions (e.g. amines, carboxylic groups) [93].

There are many ways of immobilizing enzymes through electrostatic interactions. For example, the carrier exhibits positive charges at its surface, and allows the immobilization of negatively charged enzymes. Surface may be functionalized by amino moieties (e.g. using APTES, 3-aminopropyl-triethoxysilane on silica surface) that bring positive ammoniums at the surface in acidic conditions. As a green alternative, chitosan (a chitin derivative) features amine functions at its surface [109–112], and can be deposited on solid carrier.

- Site-specific affinity attachment

A particular kind of electrostatic interactions is the “His-tag” immobilization: surfaces grafted with NTA:Ni (nitrilotriacetic acid loaded with Ni^{2+} ions) allow the formation of a strong complex with enzymes containing a poly-histidine tag (His-Tag). Histidine residues exhibit imidazole core that forms metallic complex bound with nickel atom. Through genetic engineering, His-Tag can be added to the N- or C-terminus of any enzyme and, in most cases, the modification does not impair the activity of the enzyme.

- Adsorption assisted by hydrophobic interactions

Materials exhibiting hydrophobic functions (e.g. aliphatic carbon chains, aromatic residues) or functionalized by hydrophobic functions can favour the immobilization of enzymes which exhibit important hydrophobic patches at their surface [113]. For example, silica can be functionalized by OTMS (octyltrimethylsiloxane) for the grafting of octyl moieties [113]. Controlled hydrophilicity/hydrophobicity has been demonstrated to govern enzyme immobilization [114]. In this work, we studied the immobilization of transaminase (hydrophobic residues represent more than two-thirds of its surface) on MTES functionalized silica. MTES (methyltriethoxysilane) is a hydrophobic organosilane that provides methyl functions on silica surface (*vide infra*).

- Covalent grafting

The covalent grafting of an enzyme on a support allows the strongest immobilization. However in the case of a subsequent irreversible deactivation of enzyme, both the enzyme and the support become unusable (and the support can also be expensive!) [93].

Classically, a covalent bond is formed between the enzyme and the surface (eventually functionalized). For example, covalent grafting can be based on the reaction between an amino moiety and an aldehyde function. Typically, enzymes that exhibit lysine residues at their surface (aminated amino acids) react with glutaraldehyde (bi-aldehyde agent), to form imine covalent bonds between enzyme and glutaraldehyde linker [115]. In the other side of glutaraldehyde, a second aldehyde function can react with amino moieties grafted on the carrier (e.g. silica functionalized with APTES). For a more stable bond, imine reduction is often conducted with sodium cyanoborohydride, leading to a non-hydrolyzable amine bond [116] (Figure I - 20).

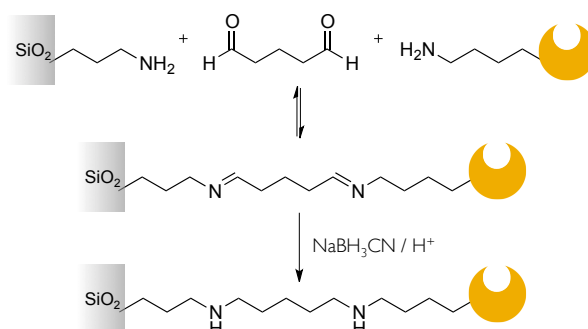


Figure I - 20 – Covalent immobilization using glutaraldehyde as linker. Reaction between amines and aldehyde moieties leads to the production of imines functions that can be reduced using cyanoborohydride forming irreversible amines.

Another typical way to perform the covalent grafting of enzyme is the use of epoxide-functionalized surfaces that react with amino-functions of the enzyme surface, forming amino-alcohols. Epoxides are brought to silica surface, using GLYMO (3-glycidyloxypropyl-trimethoxysilane) as functionalizing agent (see Chapter IV).

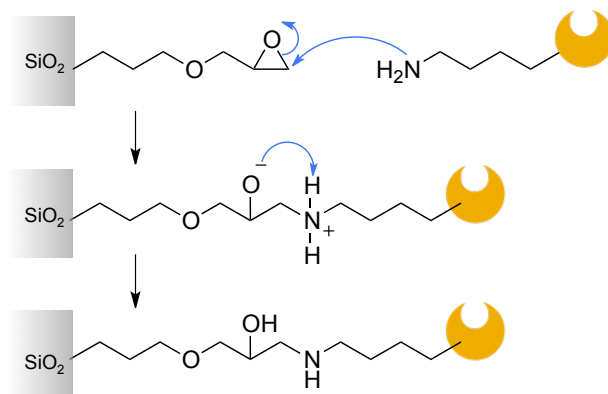


Figure 1 - 21 – Covalent immobilization of enzyme on epoxide-functionalized silica.

2.3.2.2 Entrapment

In the entrapment method, enzyme inclusions are dispersed in a matrix. The holding material (a polymer or an inorganic material) is synthesized or assembled in the presence of the enzyme [93]. Thus, this method sometimes leads to a strong enzyme deactivation [117]. The main advantage is that the enzymes are easily dispersed through the material, and are not interacting with external interfaces that could deactivate them (*e.g.* air bubbles, stirring devices) [96]. Weak physical interactions maintain the enzyme into the porous carrier. However, if the enzyme is small, a leaching is often observed. Thus, the entrapment is often combined with covalent grafting methods [93].

2.3.2.3 Cross-linking

Enzymes can form insoluble structures when they are in aggregate or crystal states. In such cases, small soluble enzymes are transformed into larger objects, which can be easily separated from the reaction medium. Thus, in the same way as immobilization onto a support or into a matrix, cross-linking can generate heterogeneous biocatalysts.

Cross-linked Enzyme Crystals (CLEC) consist in chemically cross-linked enzyme crystals [118]. They allow a total control on particle size [98]. However, crystals are hard to obtain, as it requires pure enzyme solution. Cross-linked Enzyme Aggregates (CLEA) [119] are obtained by the precipitation of native enzymes (*e.g.* using salts), followed by aggregation by a bi-functional agent (*e.g.* glutaraldehyde).

CLEC and CLEA provide excellent productivity as no extra-cost for the carrier is needed, and the total catalyst mass involved in the system is low. The enzyme concentration in CLEC and CLEA is high as compared with carrier-bound or carrier-entrapped enzymes. CLEC and CLEA often exhibit excellent stability (no denaturation by a carrier, the direct environment is composed by similar enzymes). However, in some cases, diffusional limitations into the aggregate can hamper the diffusion of substrate and product, and thus, decrease the overall relative enzymatic activity. Also, the binding of enzyme can, in some cases, deactivate the enzyme [93,97,98].

2.3.3 Carriers

There are four kinds of materials that are used as enzyme carriers [120]: (i) inorganic materials (e.g. silica, alumina, clays and zeolites); (ii) organic or synthetic materials (polymeric resins), (iii) biological materials (biopolymers such as chitosan and cellulose), and (iv) hybrid materials (a combination of previous materials).

The different categories of support materials have respective pros and cons. Inorganic materials exhibit a high specific surface area and good mechanical strength. They are inert, non toxic and the surface chemistry can be tuned by functionalization process (*vide infra*) [104,121]. Organic materials often intrinsically exhibit chemical functions at their surface, which can be used as anchoring points. They are available in wide range of composition. However, they are often vulnerable to organic solvents [93]. Biological materials are cheap and exhibit a wide range of chemical functions at their surface. However, it is hard to know or predict the surface chemistry and the distribution of chemical functions at the surface. They also have a relatively poor chemical and mechanical strength [108,110]. Considering the diversity of options available, the choice in enzyme carriers depends on several parameters, as discussed below.

(i) Surface composition of the carrier. Some materials exhibit specific chemical functions at the surface that can help immobilization. For example, the presence of oxirane functions on Eupergit®C [93,122], an acrylic resin, can be used for the covalent grafting of enzymes (that also exhibit amino moieties at their surface). The presence of hydrophobic functions is fundamental for adsorption and catalytic activity. Lipase, a model enzyme for enzyme immobilization, shows a higher activity when immobilized on hydrophobic materials. In fact, the enzyme looks like a can with a hydrophobic lid, which protects the active site. When the enzyme is immobilized on hydrophobic support, the lid opens faster, thanks to

hydrophobic interactions. Thus, the activity of lipase is increased [105,123]. The presence of hydrophilic functions, such as hydroxyl groups, is known to stabilize some enzymes, especially in organic solvents. Those functions provide a hydrophilic microenvironment around the enzyme. Thus, this immobilization allows to stabilize enzymes in organic media, that is useful for organic synthesis (pharmaceutical industries) [123,124].

(ii) Mechanical strength of enzyme carrier is a crucial parameter when working in flow mode, under high difference of pressure, or under vigorous stirring. Inorganic materials (such as silica) generally have excellent mechanical resistance [104,121], as compared to most biological materials.

(iii) Textural properties must be chosen with reference to the enzyme size. Porous materials are used because high specific surface area allows to immobilize more enzyme per gram of solid material, and thus, increase the overall process productivity [104]. It has been observed that small pores confer a confined environment, that can be favourable for enzyme activity (if the pore shape looks like the native enzyme shape, thus favourable interactions act all around the enzyme) [105,123]. Too small pores could however limit enzyme impregnation (due to hampered diffusion through pore size that is close to enzyme size). A non-spherical pore shape could induce a preferential orientation of the enzyme on the surface, and thus, may obstruct the entrance of the active site. Materials that allow the control on pore size and shapes are privileged [105].

(iv) The last crucial parameter is the compatibility of carrier material with the reaction conditions such as temperature, solvent, extreme pH, reactants and products.

2.3.4 Silica and the functionalization process

Among the possible supports discussed above, silica is selected in the present work and is therefore discussed here in more details. Silicon dioxide is naturally abundant (sand, diatoms [125]) and it can be synthesized in a bottom-up approach starting from silicon precursors using “sol-gel” routes which allow to control important parameters like texture, shape, surface functionality, etc. [126–128]. Usually, silicon alkoxides such as TEOS (tetraethyl orthosilicate, $\text{Si}(\text{OC}_2\text{H}_5)_4$) are employed as starting precursors. First, TEOS is hydrolysed (Figure I - 22) under acidic aqueous conditions. An acid catalyst (HA) allows speeding up the hydrolysis reaction. Depending

of the amount of water, the reaction may go to the completion, forming $\text{Si}(\text{OH})_4$ [127].

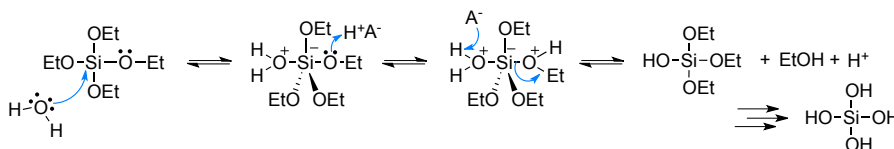


Figure I - 22 – Hydrolysis of TEOS (under acidic catalysis conditions).

Concomitantly, hydroxyl functions are condensed (Figure I - 23) together to form Si-O-Si bridges. The formed dimers then undergo further condensation reactions to yield long, interconnected silica chains. Upon further elongation and reticulation, a large silica network, called a gel, is formed along with condensation products [127]. Solvent and condensation products are trapped in the gel, generating a porous structure upon drying.

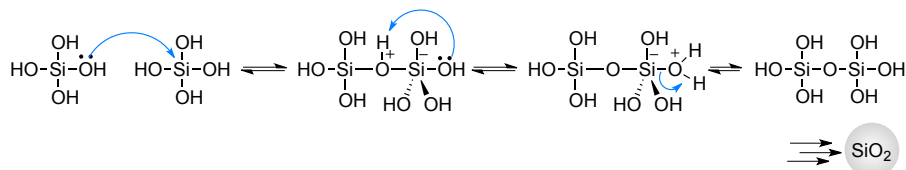


Figure I - 23 – Condensation reaction into silica. Adapted from [127].

In order to generate additional and controlled porosity in the material, it is possible to include some sacrificial templates into the sol-gel process: then, the sol-gel occurs around the templates (*e.g.* an organic phase) [129]. After the gelation, the template is removed (*e.g.* by calcination). The calcined gel keeps the shape of template imprints, which can confer a controlled texture. The sol-gel process also allows getting material with desired shapes, as the sol-gel system can be cast in any container. After hydrolysis-condensation steps, the gel takes the shape of the container.

Silica is widely used for enzyme immobilization, under various forms: powdery mesoporous silica [130–132], silica nanowires, silica monoliths [133,134], or silica network for enzyme encapsulation [135]. Silica is non-toxic, inert, and exhibits high mechanical strength.

Bare silica exhibits silanols functions at its surface. The density of silanol depends on the conditions of synthesis (concentration of water during the sol-gel process, temperature and conditions of calcination) [136]. The functionalization process allows tuning the silica surface

chemistry for example by reacting the silanol moieties with organosilanes (Figure I - 24). A base catalyst is generally employed (surface water is a suitable amphoteric catalyst for silanization) [137,138]. However, high water concentration causes the hydrolysis of organosilane that cannot react with silanol moieties anymore. Thus, water concentration should be controlled during the silanization process [139,140].

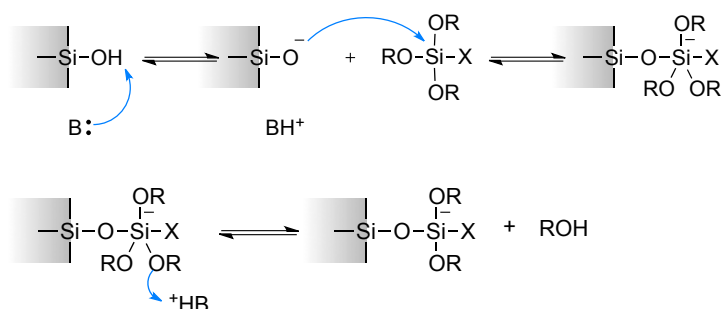


Figure I - 24 – Silanization of silica surface with organosilane. Adapted from [138].

Typical organosilanes used for surface modification and enzyme immobilization are aminated organosilanes like 3-aminopropyltriethoxysilane (APTES), methylated organosilanes like methyltriethoxysilane (MTES) and organosilanes bearing an epoxide function like (3-glycidyloxypropyl)trimethoxysilane (GLYMO) [123].

Usually, dry toluene is used as the solvent for silanization [139,141], to prevent organosilane hydrolysis. The functionalization density at the silica surface is mainly a function of the distribution of silanols (that depends on the silica synthesis process [136]). Also, the organosilane concentration may influence the silanization. For example in the case of high APTES concentrations, a lateral polymerization is observed, during which APTES molecules polymerize together instead of being individually grafted on silanol sites. This lateral polymerization phenomenon is also observed under excess of water [140,142].

A green alternative to APTES grafting (processed under organic solvent conditions) is the use of chitosan, a chitin derivative (available as a waste of sea food industry). Chitosan is a polymer of N-acetyl-D-glucosamine partially de-acetylated. It exhibits umpteen amino moieties. Amino functions are used in two purposes: first, they favour chitosan immobilization on the silica surface through electrostatic interactions between silanols (that are negatively charged) and

amines (that are positively charged) [143–145]. Second, amino functions that are not involved in the chitosan immobilization are available for enzyme immobilization (as APTES: through electrostatic interactions and covalent grafting) [110–112,146]. Immobilization of TA on chitosan was conducted in this work and reported in the Chapter II.

2.3.5 Transaminase immobilization

Owing to the huge potential interest for enzyme-catalysed synthesis of chiral amines (see Introduction, Sections 2.1.2 and 2.2.5), because of the many advantages offered by enzyme immobilization in the perspective of industrial application, the immobilization of transaminases has recently attracted growing attention. Table 1 gathers several strategies that have been applied to turn transaminases into efficient heterogeneous biocatalysts. Among all methods described in the last section, the covalent grafting, the encapsulation and the His-tag immobilization are the most employed ones. Most of the time, immobilization allowed improving the performance of the transaminase, either in terms of stability or activity.

Table 1 – Immobilization of transaminases.

Method of Immobilization	Material	Enzyme and type of reaction catalysed	Ref.
His-tag	Glass carrier	ω -TA (kinetic resolution)	[147]
Adsorption	Sepabeads® octadecyl-grafted resin	ATA-117-11Rd (asymmetric synthesis, excess of isopropyl amine and acetone removal)	[124]
Covalent grafting	Chitosan + glutaraldehyde	ω -TA (kinetic resolution)	[110–112]
Entrapment	Sol-gel matrix (+ celite)	ω -TA (kinetic resolution and deracemization)	[117,148]
CLEA / CLEC	Aggregation using glutaraldehyde	Glutamic transaminases	[149]

Yi *et al.* [111] have conducted the covalent immobilization of ω -transaminases (from *Vibrio fluvialis* JS17) on chitosan beads, using glutaraldehyde as cross-linker agent (for the covalent immobilization, and for the reticulation of chitosan beads). Residual activity of such heterogeneous catalyst reached 18 % (using a kinetic resolution reaction for enzyme activity assay). Similarly, Mallin *et al.* [110,112] have recently covalently immobilized ω -transaminases (from *Giberella zeae*, *Neosartorya fischeri*, *Aspergillus fumigatus*, *Ruegeria pomeroyi* and *Rhodobacter sphaeroides*) on chitosan beads, using glutaraldehyde as crosslinking agent. In the kinetic resolution mode, a high thermal stability was observed, along with a shift in pH optimum. A huge residual activity (359 % as compared to the activity in the same conditions, set at 100 %) was observed for enzymes that were heated before and after immobilization.

Encapsulation of ω -transaminases (ATA-113, ATA-117 and transaminase from *Vibrio fluvialis*) was successfully performed by Koszelewski *et al.* [117], using a sol-gel matrix (with celite inclusion as porous additive). Encapsulated enzymes exhibited activity even at pH 11 (whereas free enzymes were strongly deactivated in such extreme conditions), and enzymes were stable (over 5 cycles), and still enantioselective (enantiomeric excess was over 99 %). Transamination reactions were performed both in the kinetic resolution mode, and in the deracemization (one pot two steps) mode using lactate dehydrogenase or alanine dehydrogenase for driving equilibrium. This system was used to demonstrate the biocatalytic synthesis of pharmaceutically relevant chiral amines (S-Mexiletine and S-4-phenyl-2-butylamine). Pääviö *et al.* [148] recently performed the kinetic resolution of racemic amines, using ω -transaminase (from *Arthrobacter* sp.) encapsulated in sol-gel matrix (with controlled hydrophobicity, using MTES during the sol-gel synthesis). Catalysts were shown to be reusable after 5 cycles.

Recently, Cassimjee *et al.* [147] achieved the immobilization of *Chromobacterium violaceum* ω -TA through His-tag, on a glass carrier, derivatized with cobalt ions (EziG™ support). The catalyst was active under organic conditions (methyl *tert*-butyl ether as solvent) and at 50 °C. Methylbenzylamine was used as amino-donor in the kinetic resolution mode, for the amination of 1-phenoxypropan-2-one.

Hydrophobic interactions for immobilization enhancement was exploited by Truppo *et al.* [124] for the immobilization of engineered ATA-117-11Rd transaminase on Sepabeads® resin EXE 120 (polystyrene resin grafted with octadecyl moieties). The solid

biocatalyst was active in organic medium (water saturated isopropyl acetate as solvent), at high temperature (up to 60 °C) for the asymmetric synthesis of Sitagliptine. To reach high product yield, they used a large excess of isopropyl amine as amino-donor and shifting the equilibrium by further converting acetone as proposed by Savile *et al.* [7].

To our knowledge, a simple physical adsorption was never reported as a successful immobilization strategy for transaminase. Concerning the immobilization through CLEA and CLEC formation, Patramani *et al.* [149] have tried to form transaminases (glutamic-transaminases) aggregates using glutaraldehyde as cross-linking agent, for antibody isolation. However, the CLEA were not active anymore after reticulation.

2.4 Flow chemistry

2.4.1 Towards a gain in productivity

In general, batch processes involve significant maintenance time after each synthesis (time for washing and reconditioning the reactor for the next synthesis). Also, the parameters of the reaction – in particular product and reagent concentrations – evolve with time, which implies that the catalyst is not running at full speed during the whole duration of the synthesis. Flow processes allow to drastically decrease maintenance time. Flow processes allow a better control on conversion, as it is a function of reactor length (and not of reaction time, as for batch processes). Flow processes also allow for a better mixing, along with enhanced heat and mass transfers. Thus, it is generally accepted that flow processes permit to reach a high productivity level [150,151]. In the case of cascade reactions (see Section 2.2.4.3), intermediate compounds are produced in a flow reactor, whereas the starting substrate is not totally converted. Then, intermediates compounds can be involved in the next reaction. It results in a higher selectivity for such flow reactions [152].

Therefore, if one aims at the design of a truly green and industrially practicable biocatalytic process, the immobilized enzyme should be implemented in the continuous flow mode. In fact, the conversion of a batch process to a continuous flow process is often the essential link between laboratory scale and industrial scale [150].

There are mainly three kinds of flow reactors (Figure I - 25): the Continuous Stirred Tank Reactor (CSTR): an inflow is continuously fed into a stirred vessel, while an outflow is continuously removed

from the stirred vessel. If an infinite of small CSTRs were connected in series, the reactor would be similar to a Plug Flow Reactor (PFR). In such reactor, the conversion rate is depending on the residence time of substrates in the reactor, and thus depending on the flow rate. Finally, a Fixed-Bed Reactor (FBR) is a PFR where the flow is passing through a solid phase (that may contain a catalyst) [153].

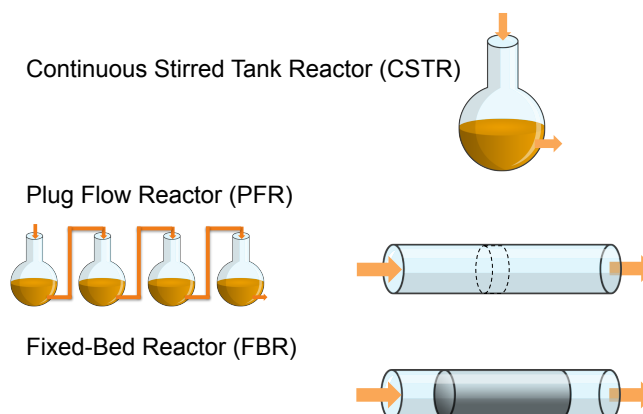


Figure I - 25 – Different kinds of flow reactors.

Catalytic efficiencies of flow processes depend on product concentration at the outlet, flow rate through the reactor, and mass of catalyst in the reactor. Comparison between batch and flow processes can be conducted using the relations shown on Figure I - 26 [150]. Usually, as flow processes have better mixing and mass and heat transfers, the reaction rate of flow processes are higher (generally 2 or 3 times higher than batch reaction time).

$$r_{\text{Batch}} = \frac{n_{\text{Product}}}{t_{\text{Batch}} \times m_{\text{Cat}}} \quad [\text{mol}_{\text{Product}} \cdot \text{s}^{-1} \cdot \text{g}_{\text{Cat}}^{-1}]$$

$$r_{\text{Flow}} = \frac{C_{\text{Product}} \times \text{Flow rate}}{m_{\text{Cat}}} \quad [\text{mol}_{\text{Product}} \cdot \text{s}^{-1} \cdot \text{g}_{\text{Cat}}^{-1}]$$

Figure I - 26 – Calculations of catalytic efficiencies. Catalytic efficiencies of batch and flow processes expressed as reaction times (r); n is the number of mole, C is the concentration, t_{Batch} is the duration of batch reaction.

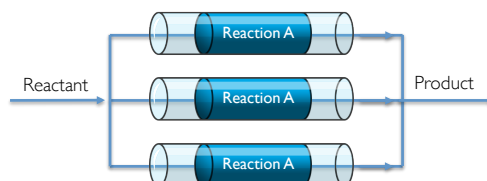
Among the advantages of flow chemistry, is the possibility to combine several reactors (Figure I - 27). When flow reactors are

connected in series, it allows increasing the global reaction length and thus, the easy increase in conversion level. Connecting reactors in parallel allows the scale-up of the process [151]. Also, flow chemistry is of great interest for multi-step reactions: in batch, when a reaction is generating an intermediate, it should be isolated to be used in a second step. However, some intermediates are dangerous to handle. In flow mode, when reactors are connected in series (each reactor handles a given reaction step in the best conditions), intermediates do not need to be isolated as they are directly fed in the next reactor. Finally, flow reactors can be combined with other processing units, such as solvent switches, purification column for by-product elimination, or mixing devices.

Reactors connected in series – control on conversion rate



Reactors connected in parallel – easy scale-up



Multi-step reactions – no intermediate isolation

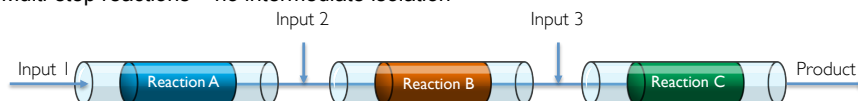


Figure I - 27 – Useful combinations of flow reactors.

One interesting application of flow chemistry is the flow chiral resolution catalysed by enzymes. An example is provided in Figure I - 28 [154]. Enantioselective acylases were immobilized on a packed-bed reactor to catalyse the de-acetylation of one enantiomer of a racemic mixture into aminated compound. A flow of hydrochloric acid is quenching the product after the enzymatic step. Thus, the aminated product is protonated, while the unreacted enantiomer is uncharged. A flow-microextractor is connected at the outlet: an organic phase is extracting the unreacted enantiomer (that is the product of interest), using ethyl acetate, while the charged amine product remains in the aqueous reaction medium. Finally, the unreacted product is easily purified, and is obtained with high enantiomeric excess (> 99 %).

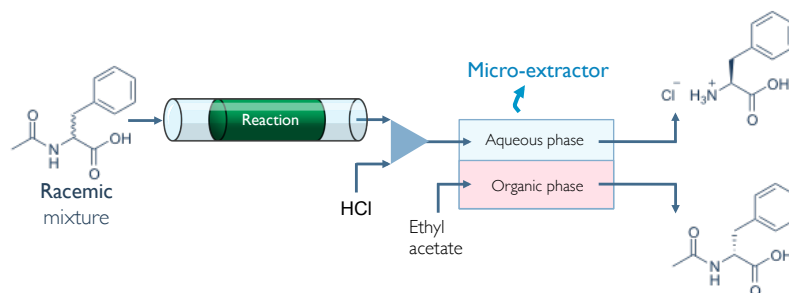


Figure I - 28 – Flow chiral resolution by acylases. Adapted from [154].

2.4.2 On the use of monoliths

Usually, batch experiments are conducted using powders as heterogeneous catalysts (for example, as carriers for enzyme immobilization). They can be easily separated from the reaction medium at the end of the reaction (by filtration or centrifugation). A drawback of the use of powders in batch reactors, however, is the attrition phenomenon: under the effect of stirring, the granulometry of the solid particles can be significantly reduced, which poses a problem for the subsequent filtering or centrifugation. Also, the immobilized enzymes could be damaged [155].

When using flow reactors with a heterogeneous catalyst, one option is to pack the powder in fixed bed configuration. The randomly packed solid bed allows avoiding attrition issues. However, if the bed has low void fraction (especially when it is filled with small catalyst particles), the pressure drop in the reactor is huge, and must be compensated by high-pressure pumps that are expensive. Thus, powdery catalysts are usually shaped into pellets or extrudates, which is not always suitable for solid biocatalysts.

Another possibility is to use self-standing and macroscopic porous materials, which allow the easy flow of liquid mixtures. The use of such monoliths instead of packed powders offers two main advantages: the easy implementation (the monolith is a one-piece object), and the high void fraction (allowing the decrease of pressure drop). Monoliths are considered as the ideal technical solution for flow processes [150,156].

Recently, an innovative sol-gel method has been developed for the production of highly porous silica monolith named “Si(HIPE)” (Silica High Internal Phase Emulsion) [157]. During material synthesis (Figure I - 29), an oil-rich emulsion is cast into a vessel, the geometry of which will determine the geometry of the monolith, thus allowing a

wide range of shapes. The obtained macrocellular silica monoliths are particularly suited for the development of continuous flow reactors [133,158]. Si(HIPE) monoliths have high void fraction allowing minimizing pressure drop. They are easier to manipulate as compared to powders because no further shaping, tableting or extrusion steps are needed [151]. These materials are exploited in the present project. Indeed, the easy shaping and handling are highly desirable properties for the study of flow reactions. The presence of many interconnected macropores allows (i) taking benefit from a relatively large surface for enzyme immobilization; (ii) the liquid flow to pass easily through the pores; and (iii) good hydrodynamic properties (turbulent flow) for efficient mixing (facilitating heat and mass transfers throughout the monolith). Thus, all those features make the Si(HIPE) monolith an ideal candidate as carrier for flow enzymatic reactions.

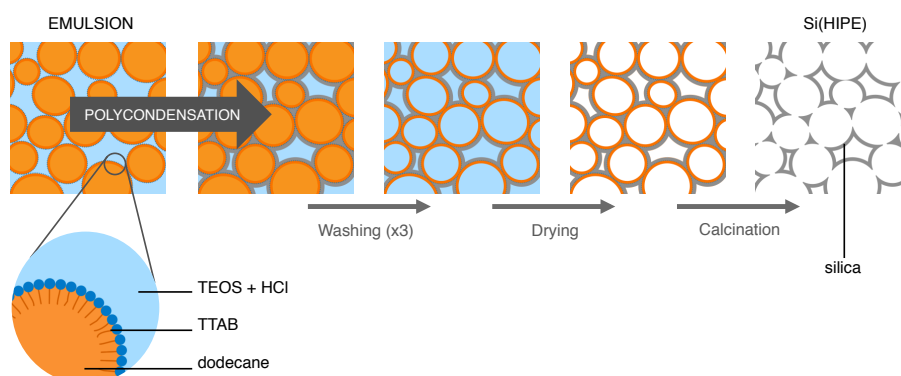


Figure I - 29 – Si(HIPE) monoliths preparation. From left to right: a surfactant-stabilized emulsion is formed between an aqueous and an organic phase. Emulsion is left to rest for one week to allow the poly-condensation of the silica network. Then three consecutive washing steps allow organic phase replacement by a volatile solvent. Drying step enables solvent evaporation. Finally, calcination step allows burning organic residues and surfactant, and strengthening the silica network. TEOS is tetraethyl orthosilicate, the silica precursor; TTAB is trimethyltetradecylammonium bromide, the surfactant.

Transaminations in continuous flow

Table 2 collects the reported studies in which transamination reactions have been performed in flow mode.

Table 2 – Transaminations in continuous flow.

Immobilization	Materials	TA	Reaction	Ref.
Encapsulation	Ca-Alginate beads	Whole cells (<i>Vibrio fluvialis</i> JS17)	Kinetic resolution (continuous removal of ketone)	[76]
Covalent grafting	Silica capillary + APTES + Glutaraldehyde	Glutamic-pyruvic transaminase, glutamate dehydrogenase	Asymmetric synthesis	[87]
His-tag immobilization	Silica capillary + Ni ²⁺ derivatization	Transaminase and transketolase	Kinetic resolution	[159]
His-tag immobilization	Agarose beads + Ni ²⁺ derivatization	Transaminase and transketolase	Kinetic resolution	[160]
Encapsulation	Methacrylate beads	Whole cells (<i>Escherichia coli</i>)	Asymmetric synthesis (favourable thermodynamics)	[161]
Encapsulation	Chitosan	Whole cells (<i>Escherichia coli</i>)	Asymmetric synthesis (continuous removal of product, using SLM)	[162]
Encapsulation	Lentikats® (polyvinyl alcohol gel)	ω-TA	Kinetic resolution	[163]

His-tag immobilization	Sepabeads® + Co ²⁺ derivatization	<i>Halomonas elongata</i> ω-TA	Asymmetric synthesis (favourable thermodynamics)	[71]
Covalent grafting	Silica monolith + APTES + glutaraldehyde	ATA-117	Kinetic resolution and asymmetric synthesis (using LDH and GDH)	This work and [1]
Covalent grafting	Epoxy resin Sepabeads®	<i>Halomonas elongata</i> ω-TA and Horse liver alcohol dehydrogenase	Kinetic resolution (followed by aldehyde reduction into alcohol)	[164]

Shin *et al.* [76] were the first to report a transamination in flow mode. Whole cells of *Vibrio fluvialis* JS17 containing overexpressed transaminases were entrapped in calcium-alginate beads (biological polymer), and used to form a packed-bed reactor. As compared to the cell-free extract, change in pH optimum was observed (from 9 to 8) and a lower substrate and product inhibition when the whole cells were used in the fixed bed process. They successfully perform the kinetic resolution of methylbenzylamine, using alanine as amino-donor. Ketone product (acetophenone) was continuously removed using a hydrophobic membrane contactor.

In 2009, Li *et al.* [87] reported on the use of flow transamination, in combination with a flow glutamate dehydrogenation, for the blood glutamate determination. Glutamic-pyruvic transaminase was immobilized into a glass micro-capillary, functionalized with APTES, and pre-activated with glutaraldehyde. Transaminase was used as a cascade enzyme for the recycling of α-keto-glutarate into glutamate (the main reaction is the glutamate dehydrogenation). As the transaminase catalyses the reaction between natural amino acid and α-keto acid, the chemical equilibrium was highly favourable.

Silica micro-reactors were also handled by Matosevic *et al.* [159] for the co-immobilization of transaminase and transketolase enzymes, and exploited for the flow synthesis of chiral amino-alcohol (2-aminobutanetriol). Nickel derivatized-silica (in combination with MTES grafting) was employed for His-tag immobilization. Transamination was conducted in the kinetic resolution mode, using methylbenzylamine as amino-donor.

Similarly, Halim *et al.* [160] also achieved the flow synthesis of amino-alcohols using transaminase and transketolase. Enzymes were immobilized through His-tag, on agarose beads derivatized with nickel-NTA. Using methylbenzylamine as the amino-donor, they successfully achieved the enantioselective synthesis of 2-amino-1,3,4-butanetriol (a building block used in pharmaceutical industry), reaching 83 % conversion after stabilization (after 20 minutes on stream).

More recently, Andrade *et al.* [161] reported on the flow transamination for the thermodynamically favourable asymmetric synthesis of non natural amines in organic solvent (methyl *tert*-butyl ether). Using *Escherichia coli* whole cells containing over-expressed R-selective transaminases and PLP cofactor were entrapped on methacrylate beads. R-Mexiletine was synthesized in 94 % yield using isopropylamine as the amino donor (residence time was 60 min). PLP leaching was avoided thanks to the organic solvent (the cofactor stayed in the aqueous phase entrapped in the beads). The biocatalysts were stable up to 10 days.

Escherichia coli whole cells containing over-expressed ω -TA from *Arthrobacter citreus* were entrapped in chitosan matrix and placed in a packed-bed reactor by Rehn *et al.* [162] for the asymmetric synthesis of S-methylbenzylamine. The equilibrium was displaced using a continuous extraction of the amine product using an SLM contactor (*i.e.* a porous membrane, with pores filled with undecane allowing the transfer of the amine). This system led to 98 % conversion in flow mode. Final product concentration was high (55 g/L obtained by processing the flow setup for 80 h).

In 2017, Bajic *et al.* [163] successfully achieved the flow transamination (kinetic resolution) of methylbenzylamine and pyruvate using ω -transaminases encapsulated in gel matrix (Lentikatz®, a polyvinyl alcohol matrix) placed in packed-bed reactor. After 21 days of process, the catalyst still exhibited up to 80 % initial activity.

Metal-derivatized epoxy-resin was used by Planchestainer *et al.* [71] for the continuous flow transamination, using *Halomonas elongata* ω -transaminases (HEwT), immobilized through His-Tag binding. The asymmetric synthesis of *p*-NO₂-benzaldehyde into *p*-NO₂-benzylamine was successfully performed (> 99% yield in 2 minutes residence time) and coupled to on-line product purification device. The amino-donor was alanine.

As described in details in the Chapter I, we recently reported on the use of ATA-117 transaminase, covalently immobilized on silica monolith -Si(HIPE)- for the flow kinetic resolution of racemic amines [1].

In 2018, Contente *et al.* [164] reported on the use of HEwT immobilized on epoxy resin, for the flow production of aromatic primary alcohol, starting from aromatic amines (transamination was followed by reduction of aldehyde product into alcohol of interest). For example, starting from dopamine (biologically available), they successfully synthesized hydroxytyrosol (an antioxidant compound), with high yield (82 %) and excellent enantioselectivity. Their flow setup was connected to on-line purification device, allowing cofactor recovering and product purification.

3 Objectives

The general aim of this PhD thesis is the development of efficient heterogeneous biocatalysts for the green production of industrially relevant chiral amines in continuous flow mode. As mentioned in the previous section, the remaining challenges are (i) the displacement of the chemical equilibrium towards the asymmetric synthesis of industrially relevant chiral amines, (ii) the controlled immobilization of transaminases, and (iii) the production of such chiral amines in continuous flow.

The following specific objectives have been set in order to fulfil that aim:

- The synthesis of structured solid biocatalysts by determining optimal conditions for their controlled development (silica functionalization process, enzyme immobilization);
- The implementation of those biocatalysts in an optimized flow setup (by determining flow and reaction parameters) in order to assess their efficiency;
- The development of a multi-enzymatic cascade reaction in flow mode in order to produce chiral amines by asymmetric synthesis;
- The transfer of this technology for the flow production of other organic compounds of relevant interest, using other immobilized enzymes.

4 Strategy

4.1 The project

This project proposes the controlled immobilization of enzymes on Si(HIPE) monoliths for the continuous flow production of chiral amines (Figure I - 30).

The transaminase ATA-117 is first studied as a free homogeneous biocatalyst in a batch reactor to determine kinetic parameters and optimal operating conditions. Then, several immobilization methods are tested (simple adsorption, adsorption assisted by hydrophobic and/or electrostatic interactions, covalent grafting, His-tag immobilization) on the silica functionalized by methyl, epoxy groups or amine functions (respectively, silanization by MTES, GLYMO or APTES). Covalent grafting is performed either by using glutaraldehyde as a coupling agent on aminated silica and enzyme, or by coupling epoxy functions with amino moieties on the enzyme surface. A greener alternative is also considered: the use of chitosan, a green biopolymer, as a substitute to the APTES grafting. Immobilization modified enzymes using specific tag (e.g. enzyme containing His-Tag) is also considered.

Characterization of functionalized monoliths are carried out to reach a fine understanding of mechanisms controlling the functionalization step: nitrogen physisorption, scanning electron microscopy, thermogravimetric analysis, infrared spectroscopy, thermo-programmed water desorption and thermo-programmed calcination.

At first, the immobilized transaminases are tested in the model reaction of kinetic resolution of a racemic mixture of *p*-bromo-methylbenzylamine (BMBA). This reaction consumes a chiral amine, and may therefore appear economically irrelevant but (i) allows us assessing enzyme activity in the elementary transamination act since it is not limited by the thermodynamic equilibrium [148,165], and (ii) is relevant in the way of production of complex ketones, that could be reduced and valued for the production of chiral alcohols (starting from complex amines) [164].

After setting the proof-of-concept for flow transamination reactions using transaminase immobilized on silica monoliths, we propose the implementation of the multi-enzymatic system « ATA-YADH-GDH » for the asymmetric synthesis (Figure I - 30). For each enzyme, a model reaction is initially studied in order to assess biocatalysts

efficiency, first in batch (determining operating conditions, stability), then in flow conditions (determining optimal immobilization conditions). In that purpose, each enzyme is studied first in homogeneous batch. At the end, a common operating window will be established for all immobilized enzymes. Flow parameters (diffusional limitations) will be also studied.

Finally, other organic compounds of relevant interest will be synthesized in flow in order to demonstrate the applicability of the technology for other enzyme-catalysed reactions.

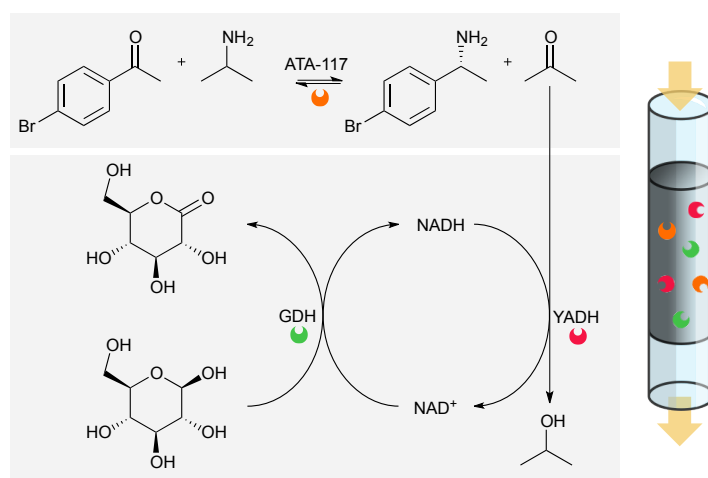


Figure I - 30 – The project. We propose the implementation of multi-enzymatic cascade reactions for chiral amines production implemented in flow mode. ATA-117 is the transaminase, YADH is the Yeast Alcohol Dehydrogenase, and GDH is the Glucose Dehydrogenase.

A cyclic research strategy is implemented (Figure I - 31): Biocatalysts are synthesized by immobilizing transaminases on a solid support; they are characterized in the course of their development; their efficiency is assessed by a flow transamination reaction. This cyclic strategy is repeated by considering previous results to improve the system.

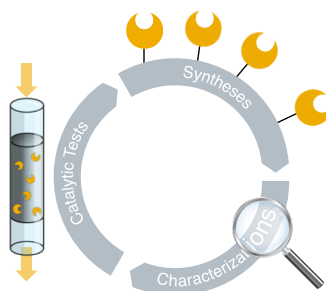


Figure I - 31 – Cyclic strategy implemented. Syntheses of biocatalysts, characterizations and catalytic tests.

4.2 Work plan

Several interconnected lines of research are conducted in parallel (Figure I - 32).

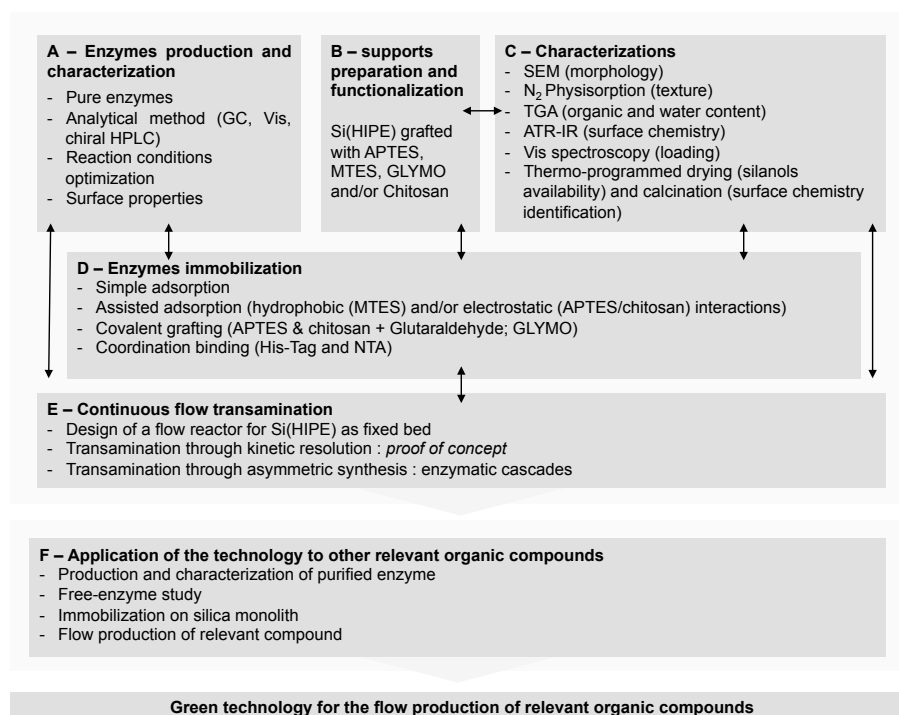


Figure I - 32 – Work plan.

A – Enzymes production and characterization

Either enzymes are commercially available, or they are produced (through microbial transformation, expression and purification) to obtain free enzymes.

Studying free enzymes allows choosing suitable immobilization strategies, and identifying optimal operating conditions in the homogeneous phase to ensure that the best conditions are satisfied in heterogeneous phase: (i) analytical methods are optimized for enzyme quantitation (Vis spectroscopy), reaction kinetics monitoring (solvent extraction and detection by gas chromatography (GC), or Vis spectroscopy), and chiral detection (extraction and detection by high performance liquid chromatography, HPLC). (ii) Optimal operating conditions are determined by varying the working pH, reactants and products concentrations. Enzyme stability over time and storage is studied. (iii) Enzyme surface properties (from protein sequences and crystallographic data) allow selecting suitable immobilization strategies (isoelectric point, hydrophobic patches).

B - Support preparation

In this part of the project, suitable carriers for enzyme immobilization and flow reactions are prepared: (i) Si(HIPE) monoliths are synthesized (cylindrical geometry, convenient for flow chemistry), (ii) and functionalized (optimized by varying MTES, APTES, GLYMO and chitosan concentrations, working pH, incipient wetness impregnation or wet impregnation, vacuum, water content).

C - Characterizations

An accurate characterization of biocatalysts is carried out at each stages of their development: (i) Scanning electron microscopy (SEM, materials morphology), (ii) nitrogen physisorption (textural properties), (iii) thermogravimetric analysis (TGA, organic functions and water content), (iv) infrared spectroscopy (ATR-FTIR, surface chemistry), (v) visible spectroscopy (Vis spectroscopy, enzyme loading), and (vi) thermo-programmed water desorption (silanols availability) and calcination (identification of organic matter).

D - Enzymes immobilization

Enzymes are immobilized either by adsorption (simple, assisted by MTES, APTES or chitosan), by covalent grafting (GLYMO, APTES or chitosan, amine moieties pre-activated by glutaraldehyde) or by

site-specificity affinity attachment (His-Tag and NTA moieties). For each biocatalyst, concentration of in- and outflows are determined, in order to establish the loading in the monolith (by a mass balance, detection by Bradford method).

E - Continuous flow transaminations

Continuous flow transamination allows to assess biocatalysts efficiency: (i) suitable flow reactor for Si(HIPE) geometry is designed. (ii) Kinetic resolution transamination reactions are conducted to demonstrate the feasibility of the concept. (iii) Multi-enzymatic cascade reactions (cooperation between ATA-117, YADH and GDH) allow producing chiral amines. First, each enzyme are independently immobilized on Si(HIPE) to determine a mutual acceptable operating windows for the immobilization of all enzymes and the flow transamination. Then, the three enzymes concentration ratio is studied in order to adjust the relative rates and affinities for the support. Indeed, those parameters may not be identical for each of the three enzymes. In that case, specific rate for one enzyme is estimated by injecting only specific reactants for that enzyme.

F – Application of the technology to the flow production of other relevant organic compounds

The same strategy (from A to E) is repeated and applied to another interesting chemical reaction, *i.e.* the synthesis of coumaric acid from tyrosine. A Tyrosine ammonia-lyase (TAL) is produced and characterized to determine the most suitable immobilization method. Then, enzymes are immobilized and used as a catalyst for flow production of coumaric acid. The aim of this last step is to demonstrate the applicability of the technology to larger range of reactions and enzymes.

Experimental

1 Materials

Acetone ($\geq 99.9\%$), 3-aminopropyl-triethoxysilane (APTES; $\geq 98\%$), ammonium persulfate (APS, $\geq 98\%$), Na,N α -bis(carboxymethyl)-L-lysine (Lys-NTA; $\geq 97\%$), Brilliant blue G 250, 4'-bromoacetophenone (BAP; $\geq 98\%$), R-4-bromo- α -methylbenzylamine (R-BMBA, 99 %), S-4-bromo- α -methylbenzylamine (S-BMBA, 99 %), bromophenol blue, diethylamine (HPLC grade), n-dodecane ($\geq 90\%$), ethylenediaminetetraacetic acid (EDTA, $\geq 99\%$), hydrobromic acid (48 %wt, aqueous solution), hydrochloric acid (37 %wt, aqueous solution), isopropylamine ($\geq 99.5\%$), LB-Agar Medium (Lysogeny Broth, LB), magnesium (II) chloride hexahydrate ($\geq 99\%$), phosphoric acid aqueous solution (85 %wt, aqueous solution), pyridoxal 5'-phosphate hydrate (PLP; $\geq 98\%$), Super Optimal Broth medium (SOC medium), sodium cyanoborohydride solution (5.0 M in 1 M NaOH; NaBH₃CN), sodium hydroxide aqueous solution (50 %), sodium pyruvate ($\geq 99\%$), Terrific Broth medium (TB medium), tetraethyl orthosilicate (TEOS; $\geq 98\%$), toluene ($\geq 99.8\%$, anhydrous), triethoxymethylsilane (MTES, 99 %), trimethyltetradecylammonium bromide (TTAB; $\geq 99\%$), L-tyrosine methyl ester (Me-TYR), o-xylylenediamine dihydrochloride (XDD, $\geq 98\%$), and Yeast Alcohol Dehydrogenase (YADH, from *Saccharomyces cerevisiae*, lyophilized powder) were purchased from Sigma-Aldrich. Glycerol ($\geq 99\%$), glycine ($\geq 99\%$), imidazole ($\geq 99.5\%$), lysozyme from chicken egg white (lyophilized powder, ≥ 40 kU/mg), 3-morpholinopropano-1-sulfonic acid (MOPS, 99.5 %), sodium dodecylsulfate (SDS, 98.5 %), trizma®Base (Tris base, 99 %) were purchased from Sigma Life Science. DNase I (grade II), His-Tag purification resin, kanamycin sulfate, proteases inhibitor cocktail were purchased from Roche. 4-bromo- α -methylbenzylamine (BMBA; $\geq 99\%$; racemate), crystal violet (pure), dibasic potassium phosphate ($\geq 99\%$), dimethylsulfoxide (DMSO; $\geq 99.8\%$) and monobasic potassium phosphate ($\geq 99\%$) were purchased from Acros Organics. Acrylamide mix (40% aqueous solution; acrylamide : bisacrylamide ratio is 29 :1), agarose-His/Ni Beads (Roti®garose), D-Alanine ($\geq 99\%$), ethylamine hydrochloride ($\geq 98\%$), glacial acetic acid, D-(+)-Glucose ($\geq 99.9\%$), isopropyl alcohol ($\geq 99.5\%$), Lactate dehydrogenase from porcine heart muscle (LDH, from *Sus scrofa domestica*, lyophilized powder), Lysogeny broth medium (LB medium), methanol, β -Nicotinamide adenine dinucleotide (NAD⁺, 98 %), β -Nicotinamide adenine dinucleotide disodium salt (NADH ≥ 98

Experimental

%), nickel (II) chloride hexahydrate ($\geq 98\%$), sodium carbonate (Na_2CO_3 , $\geq 99.5\%$), sodium chloride (NaCl , $\geq 99\%$), sodium hydroxide (NaOH , $\geq 99\%$), tris(hydroxymethyl)aminomethane hydrochloride (TRIS-HCl, $\geq 99\%$), urea, were purchased from CarlRoth. L-tyrosine ethyl ester (Et-TYR) and ethyl coumaric ester (Et-CA) were purchased from Afla Aesar. Invisorb spin DNA extraction kit was supplied from Stratec Molecular. TALON® Superflow™ cobalt-IMAC (TALON column), PD-10 column sephadex G-25 (Desalting column) were purchased from GE Healthcare. 3-DL-Aminobutyric acid (ABA, $> 98\%$), (4-Fluorophenyl)acetone (FPA, $> 98\%$), 3-glycidyloxypropyltrimethoxy-silane (GLYMO; $\geq 95\%$), methyl coumaric ester (Me-CA), trans-p-coumaric acid (CA, $> 98\%$), L-(-)-tyrosine ($> 98.5\%$) were purchased from Tokyo Chemical Industry. Cobalt (II) nitrate hexahydrate (98.3%), dichloromethane ($\geq 99.80\%$), ethanol (99%), isohexane (HPLC grade), isopropyl alcohol (HPLC grade), tetrahydrofuran (THF; $\geq 99.6\%$) were purchased from VWR Chemicals. Dithiothreitol (DTT; molecular biology grade), One shot® BL21DE3 chemically competent *E.coli* cells, select agar, PageRuler™ stained Protein Ladder, Pierce™ BCA Protein Assay Kit, Protein prestained ProbeRuler, were purchased from ThermoFisher Scientific. GelPilot® Loading Dye 5x was purchased from Qiagen. Tetramethylethylene diamine (TEMED) was purchased from Bio-Rad Laboratories. Instant Blue™ was purchased from Expedeon. Isopropyl β -D-1-thiogalactopyranoside (IPTG) was purchased from Immunosource BVBA. Yeast extract and Tryptone were provided from Conda. Heat-shrinkable polytetrafluoroethylene (PTFE) tube was purchased from RS Components. Transaminase ATA-117 (from *Arthrobacter spp.*, see protein sequence in Section 2.2.2) and Glucose Dehydrogenase CDX-901 (GDH) (lyophilized powders) were generously supplied by Codexis. Transaminase HEwT (from *Halomonas elongata*, lyophilized powder) was graciously supplied by Prof. Francesca Paradisi (University of Nottingham, UK). Transaminase ATA-256 (lyophilized powder) was generously supplied by Dr. Elaine O'Reilly (University of Nottingham, UK). His-Tagged-ATA117 plasmid (see Annexe 7) was purchased from GeneScript. FjTAL plasmid (see sequence in Annexe 0) was graciously supplied by A. Nielsen (Technical University of Denmark, DK). Distilled water was applied for all synthesis and treatment processes.

Most experiments were conducted in the heterogeneous (bio-) catalysis laboratory at the University of Louvain (Louvain-la-Neuve, Belgium). His-tagged transaminase (His-TA) was produced, purified and characterized in the biophysics, biochemistry and microorganisms genetics laboratory at the University of Louvain

(Louvain-la-Neuve, Belgium). Tyrosine Ammonia-Lyase (TAL) was produced, characterized and purified in the Leibniz Institute of Plant Biochemistry, under the supervision of Dr. Martin Weissenborn (Halle, Germany).

2 Methods

2.1 Synthesis

2.1.1 Enzymes production

His-Tagged transaminase (His-TA) and Tyrosine-ammonia lyase (TAL) have been produced according to the same methods: (i) DNA is inserted into competent bacteria that are transformed (*i.e.* DNA of interest is incorporated in their own DNA). (ii) Enzyme of interest is overexpressed into bacteria. (iii) Enzymes (that are both his-tagged) are purified and (iv) characterized (enzyme purity and concentration determination). As the enzymes have been produced in different laboratory, slight variations on the protocols are observed.

2.1.1.1 His-Tagged ATA-117 transaminase (His-TA)

This part of the work has been entirely achieved by Ir. Adrien Gauchet during his master thesis internship.

i. Bacterial transformation

50 μ L aliquot of BL21 *E.coli* strains is mixed with 0.16 μ g plasmid, and electroporated using Bio-Rad GenePulser Xcell (2.5 kV, cuvette diameter 2 mm). Then, 1 mL LB medium is added, and the mix is incubated for 1 hour at 37 °C, and spread over petri plates, containing LB-Agar and kanamycin, using glass beads. Petri plate is incubated for 37 °C overnight.

15 mL TB medium are mixed with 15 μ L kanamycin (25 g/L) in a 50 mL culture tube (closed but oxygen permeable). This tube is inoculated with a single colony from the plate, and then incubated at 37 °C at 180 rpm overnight. Aliquots of this suspension are then mixed with glycerol (final concentration is 20 %_{vol} glycerol), flash-frozen and stored at -80 °C. Those aliquots are the transformed cells.

ii. Enzyme expression

15 mL LB medium is mixed with 15 μ L kanamycin in a 50 mL culture tube, inoculated with an aliquot of transformed cells, and incubated at 37 °C, at 180 rpm, overnight to obtain a “pre-culture”

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suspension. 500 mL TB medium is inoculated with 5 mL of pre-culture suspension in a 1L Erlenmeyer with baffles, and then incubated at 37 °C, at 180 rpm, during 2 to 4 h (in order to be in the exponential phase of the bacterial growth). This phase is reached when optical density (OD) at 600 nm reaches 0.4 or 0.5 (using Nanodrop ND-1000, Isogen Life Science). Culture suspension is then centrifuged 10 minutes at 4000 rpm at 4 °C. Supernatant is discarded, and cells are re-suspended in 500 mL fresh TB medium in 1 L Erlenmeyer with baffles. 500 µL IPTG (1 M) is added for enzyme expression induction, and then the erlenmeyer is incubated at 25 °C for 4 h at 180 rpm.

Culture suspension is centrifuged again (10 minutes, 4000 rpm at 4 °C). Cell pellet is collected and re-suspended into 20 mL phosphate buffer for cell lysis (100 mM, pH 8, imidazole 2 mM, MgCl₂ 2 mM) in 50 mL tube, and then flash-frozen and stored at -80 °C. Those tubes are the expressed proteins in whole cells.

iii. Purification

- Cell lysis

Expressed proteins in whole cells tube is thawed at room temperature in water bath. Two tablets of protease inhibitor (EDTA-free) are crushed and added to the protein tube during the thawing phase. Then 1 mL lysozyme solution (20 g/L) and 500 µL DNaseI (5 g/L) are added, and the tube is incubated on ice for 90 minutes. Then, sonication is performed (using Ultrasonic Processor 75022 VibraCell™, Sonics®) with amplitude of 70 during 10 minutes on ice. Suspension is then centrifuged at 12 000 rpm for 20 minutes at 4 °C. Supernatant is collected for the purification on column. Further steps are performed in cold room (4 °C) unless otherwise stated.

- Protein purification on-column

Disposable 5 mL propylene columns with frits (Quiagen) are filled with 2 mL resin grafted with nitrilotriacetic acid complexed with nickel atoms (NTA:Ni), and washed with 10 mL phosphate lysis buffer. Columns are then closed at the bottom-end. Lysis supernatant is filtered (on Millex®-HV membrane, 0.45 µm) and 5 mL is added on the top of the column. Columns is closed and gently shaken for 90 minutes to allow enzyme immobilization on the resin NTA:Ni beads. Column is then opened at both ends, and 2x 6 mL of washing buffer (Washing buffer, W) is flowed through the column. Enzyme elution is conducted with 6x 1 mL elution buffer (Elution Buffer, E). Each 1 mL

fraction is collected independently to determine enzyme concentration (see *infra*).

- Dialysis and ultrafiltration

Enzyme fractions are dialysed (using dialysis membrane SpectraPor®, MWCO 12000-14000, Spectrum Laboratories Inc.) for 30 minutes in 2 L phosphate buffer (100 mM, pH 8) in order to get rid of residual salts and imidazole. Enzyme solutions are then ultra-filtered in 50 mL tubes (Centrifugal Filter Units, Amicon® Ultra-15 10K, Merck Millipore Ltd) by centrifugation at 4 °C at 4000 rpm.

- iv. Enzyme characterization

- Sodium Dodecyl Sulfate – PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

Commercial gels (iD PAGE Gel 10 %, 15wells 40 µL, 10 p/k, Eurogentec) are inserted in PowerPac™ HC (Bio-Rad Laboratories) electrophoresis box containing 600 mL MOPS buffer. 2µL PageRuler™ is loaded in the reference well. Samples are prepared (8 µL enzyme sample, 2 µL GelPilot® for staining, boiling for 6 minutes) and then insterted into the other wells.

Electrophoresis is run for 25-30 minutes at 180 V (until the coloured bands reached the border between the stacking and the separating gels).

Gels are removed from the electrophoresis box and placed on a container, covered with Coomassie staining solution (Instant Blue™) under gentle shaking for 20 minutes. Then the staining solution is removed and the gel is washed with water until the gel background is clear while protein bands remain visible. Finally, the gel is scanned.

- Enzyme concentration

Enzyme concentration is measured using NanoDrop ND-1000 (Isogen Life Science) at 280 nm (using phosphate buffer as a blank). Enzyme is aliquoted in 1.5 mL tubes and flash-frozen and stored at -80 °C.

2.1.1.2 Tyrosine-Ammonia Lyase (TAL)

This part of the work has been achieved in the Leibniz Institute of Plant Biotechnology (Halle, Germany) under the supervision of Dr. Martin Weissenborn.

Experimental

i. Bacterial transformation

The first step aimed at the production of a larger amount of pure FjTAL plasmid. First, the plasmid is duplicated in DH5 α *E. coli* strain, then purified and ready for transformation and over-expression in BL21(DE3) *E. coli* strain.

A 100 μ L aliquot of competent *E. coli* cells (DH5 α) is thawed on ice for 5 minutes. 20 ng of original plasmid (FjTAL) is added, gently mixed and incubated for 15 minutes on ice. Cell suspension is heat-shocked at 42 °C for 90 seconds at 600 rpm, and then placed on ice for 3 minutes. 250 μ L of SOC medium is added and cells are incubated at 37 °C for 1 hour at 500 rpm. 100 μ L of cell suspension are then spread on petri plate containing LB-Agar medium and kamanycin. Plate is incubated at 37 °C overnight.

3 mL LB medium is inoculated with a single colony from the plate. 3 μ L kanamycin (stock aqueous solution 50 mg/mL) is added and the tube is incubated at 37 °C under vigorous shaking overnight. Plasmid extraction and purification is achieved with the plasmid purification kit (Invisorb spin DNA extraction kit, Stratec Molecular). Plasmid concentration is measured by absorbance at 280 nm using a Tecan spectrometer (Tecan Infinite F200Pro, software : Tecan i-control).

For the transformation itself, A 100 μ L aliquot of competent *E. coli* cells (BL21(DE3)) is thawed on ice for 5 minutes. 100 ng of duplicated plasmid is added, gently mixed and incubated for 15 minutes on ice. Cell suspension is heat-shocked at 42 °C for 90 seconds at 600 rpm, and then placed on ice for 3 minutes. 250 μ L of SOC medium is added and cells are incubated at 37 °C for 1 hour at 500 rpm. 200 μ L of cell suspension in then spread on petri plate containing LB-Agar medium and kamanycin. Plate is incubated at 37 °C overnight.

ii. Enzyme expression

5 mL LB medium is inoculated with a single colony (picked on the plate resulting from the transformation in BL21(DE3)). Then 5 μ L kanamycin are added. Tube is incubated at 37 °C under vigorous shaking overnight.

2 mL culture is added to 400 mL TB medium and 400 μ L kanamycin in a Erlenmeyer flask containing baffles. Flask is incubated under vigorous shaking at 37 °C till optical density (OD) at 600 nm reached 0.5 (takes typically 2.5 h). OD is measured using Ultrospec 10 from Amersham Biosciences. Then, a 250 μ L aliquot is

sampled, labelled « Before Induction » and frozen. Then 400 µL IPTG (stock aqueous solution 1 M) is added to the flask and the flask is incubated at 25 °C under vigorous shaking overnight.

iii. Purification

- Cell lysis

An aliquot is sampled from the flask, labelled « After Induction » and frozen. All the purification steps are conducted on ice and reactants are cold (unless otherwise stated). The cells are harvested by centrifugation at 4 °C, 3500 rpm for 30 minutes. The supernatant is discarded and the cells are re-suspended on binding buffer (1 volume of cell for 3 volume of buffer). Suspensions are redistributed on 15 mL aliquots. 25 mg of lysozyme and 300 µL of DNase I (stock aqueous solution 1.5 mg/mL) are added to each aliquot. After mixing, tubes are incubated on ice for 20 minutes.

Cell suspensions are then sonicated by pulsing for 30 seconds: 1 second ultrasonic pulse followed by 1 second without pulsation. Sonication is repeated 8 times interrupted by 1 minute without ultrasonic pulsing between each round.

Suspensions are then centrifuged at 4 °C for 45 minutes at 6000 rpm.

- Protein purification

TALON-column (10 mL) is first equilibrated with 10 mL cold water, then 10 mL of binding buffer. Supernatant (sampled « L ») is added to the column and the outflows is collected and labelled « FT » (for « flow-through »). The column is then rinsed with 10 mL of washing buffer and the outflows are collected (named « W »). 4 mL of Elution buffer is added to the column and outflows are collected (named « E »). The column is then cleaned with respectively 10 mL water, 5 mL NaOH (1M), 10 mL water, 5 mL Urea (8 M), 10 mL water and 10 mL of a 20 % ethanol solution in water. Column is stored at 4 °C prior to the next purification.

- Ultrafiltration

The Falcon concentrator (Protein Concentrator PES 30K MWCO) is first washed with cold water then twice with 5 mL of exchange buffer and centrifuged at 4 °C for 15 minutes at 5000 rpm. The tube « E », containing the proteins, is transferred to the Falcon concentrator. The concentrator is centrifuged for 15 minutes at 4 °C

Experimental

at 5000 rpm. Afterwards, protein solution volume typically reached 1.5 mL.

Concentration column is equilibrated with 30 mL cold water, then 30 mL exchange buffer. Then, concentrate protein solution (1.5 mL) is added to the column, followed by 1 mL exchange buffer (final volume must be set to 2.5 mL). Finally, 3.5 mL exchange buffer are added and the outflows are collected in a 5-mL tube. Tube is then flash-frozen and stored at -20 °C. Column is cleaned (30 mL water, then 30 mL ethanol solution at 20 %) and stored at 4 °C. The Falcon concentrator is washed with water and 5 mL ethanol 20 % solution and then centrifuged (15 minutes at 4°C at 5000 rpm) and stored at 4 °C.

iv. Enzyme characterization

- Protein concentration assay – BCA Assay

Protein concentration is determined through the BCA assay with Pierce BCA Assay kit. Samples are analyzed in triplicates and the finale concentration is given as the average concentration of triplicates. Absorbance at 562 nm is measured using a Tecan spectrometer (Tecan Infinite F200Pro, software : Tecan i-control).

- SDS-PAGE

Samples are mixed with the stained loading buffer (10 µL sample for 90 µL loading buffer). Samples are heated at 95 for 5 min under vigorous shaking (600 rpm).

Separating gel is prepared by mixing reactants together: 4.8 mL distilled water, 2.48 mL acrylamide mix, 2.5 mL TRIS 1.5 M buffer (pH 8.8), 0.1 mL SDS solution (10 %), 0.1 mL APS solution (10 %), 4 µL TEMED. Then the mix is poured between 2 glass plates and the phase is covered by isopropyl alcohol (forming a second phase with the gel-matrix liquid). Gelation occurs in 20 minutes. Isopropyl alcohol is removed and the stacking solution is poured over the separating gel. Stacking solution contains 1.46 mL distilled water, 0.25 mL acrylamide mix, 0.25 mL TRIS 1 M Buffer (pH 6.6), 20 µL SDS solution (10 %), 20 µL APS solution (10 %) and 2 µL TEMED. A comb is inserted into the stacking solution prior to the gelation, in order to form wells, and is removed after the gelation.

After polymerization, the gel is placed in an electrophoresis box, and covered by running buffer.

Samples and protein ladder are loaded in each well (15 μ L). Electrophoresis is run for 15 min at 90 V (until the coloured bands reached the border between the stacking and the separating gels), using the power supply Fisher scientific FB300. Then, the potential is set to 120 V, until the protein bands reach the bottom of the separating gel.

Gels are removed from the electrophoresis box and placed on a container, covered with Coomassie staining solution under gentle shaking for 15 minutes. Then the staining solution is removed and a Coomassie bleaching solution is applied until the gel background is clear while protein bands remain visible. Finally, the gel is scanned.

2.1.2 Monolith synthesis

2.1.2.1 Si(HIPE) monoliths

Porous monoliths synthesis was performed according to Ungureanu *et al.* [157]: 6 g of a concentrated hydrochloric acid solution (37 % wt) were introduced in 16 g of a TTAB aqueous solution (35 % wt). Then 5 g of TEOS was added. The aqueous phase was stirred until a monophasic hydrophilic medium was obtained (approximatively in 10 seconds). To form an emulsion, 35 g dodecane was then added dropwise in a mortar while stirring with a pestle. This step lasts approximatively 5 minutes. Emulsion was cast into a polypropylene 10 mL flask, allowed to condense for 1 week at room temperature. In order to remove residual organic compounds, resulting material was washed three times with 50 mL of a THF/acetone mixture (1:1 v/v) (each washing lasted 24 h) and then gently dried in air during 3 days before calcined at 650 $^{\circ}$ C for 6 h (heating rate of 2 $^{\circ}$ C/min with a first plateau at 180 $^{\circ}$ C for 6 h). Porous monoliths were stored in a desiccator at room temperature.

2.1.2.2 Hydrophobic monoliths synthesis by adding MTES in the emulsion (One-pot method)

Hydrophobic monoliths synthesis was adapted from the Si(HIPE) synthesis proposed by Ungureanu *et al.* [166]: 6 g of a concentrated hydrochloric acid solution (37% wt) were introduced in 16 g of a TTAB aqueous solution (35% wt). Then, MTES was mixed with TEOS in various proportion, but in any cases, 0.024 mol of total silicon was kept, and the mixture was added to the HCl/TTAB mixture.

The aqueous phase was stirred until a monophasic medium was obtained. To form an emulsion, 35 g dodecane was then added

Experimental

dropwise in a mortar while stirring. Emulsion was cast into a polypropylene 10 mL flask, allowed to condense for 1 week at room temperature. Resulting material was washed three times with 50 mL of a THF/acetone mixture (1:1 v/v) (each washing lasted 24 h) and then gently dried in air.

- Removal of surfactant with acetic acid and sodium carbonate solutions

In some cases, hydrophobic monoliths were washed with acetic acid solution using a protocole adapted from Endo *et al* [167]. In that purpose, monoliths were soaked in 50 mL of acetic acid solution (2wt%) of controlled pH (2.5 or 4) and vacuum was applied to force the solution getting through the pores of the monolith. Monoliths stayed into the solution from 24h under static vacuum. Then, they were removed and dried at 60 °C under vacuum for 24h. After, monoliths were soaked in 50 mL sodium carbonate solution (0.1 mol/L) and vacuum was applied. After 24h soaking, they were removed and dried at 60 °C for 24h under vacuum.

- Removal of surfactant by washing with ethanol

For the washing with ethanol, hydrophobic monoliths were soaked in 50 mL ethanol (99%) for 24 h under static vacuum. They were then dried at 60 °C for 24h under vacuum.

2.1.2.3 Monolith functionalization

Grafted samples are denoted Ax_Y , Mx_Y and Gx_Y where A stands for APTES grafting, M stands for MTES grafting, G stands for GLYMO grafting, x is the organosilane concentration (*i.e.* APTES, MTES and/or GLYMO) in the grafting solution, Y is the functionalization method used for the monolith (method A, B, C or D).

Method A – Soaking the monolith in the grafting solution

This protocol was adapted from Ungureanu *et al* [157]: 2 monoliths (approximately 0.15 g each) were added into 25 mL of a toluene/organosilane solution of desired concentration. Dynamic vacuum was applied to force the solution into the pores of the monoliths, until effervescence stopped. Static vacuum was then maintained for 24 h. Monoliths were separated from the toluene/organosilane solution and washed three times with 25 mL of a toluene/acetone mixture (1:1 v/v). For each washing, wet monoliths were soaked into the washing solvents by applying dynamic vacuum until effervescence stopped and a static vacuum for 2 h.

Functionalized monoliths were dried under vacuum at 60 °C for 24 h and then stored in a desiccator at room temperature.

Method B – Brief dipping, then ageing in solvent atmosphere

This protocol is similar to “Method A”, but right after the effervescence step, monoliths were removed from the grafting solution, and disposed on a grid under toluene-saturated atmosphere for 24 h (in a closed-desiccator containing a toluene flask for atmosphere saturation). They were then washed three times with 25 mL of a toluene/acetone mixture (1:1 v/v). For each washing, wet monoliths were soaked into the washing solvents by applying dynamic vacuum until effervescence stopped and a static vacuum for 2 h. Functionalized monoliths were dried under vacuum at 60 °C for 24 h and then stored in a desiccator at room temperature. Organosilane concentration used for this « dry impregnation » method is higher than the concentration used in the Method A.

Method C – Re-hydroxylating prior to silanization

A re-hydroxylation protocol was applied (adapted from Zhuravlev [136]): monoliths were soaked with water at room temperature. Dynamic vacuum was applied until the effervescence stopped. Monoliths and liquid water were then inserted in a heat and pressure-resistant glass closed-bottle, and maintained at 100 °C for 24 h. Monoliths were then dried at 190 °C for 24 h under vacuum on a glass petri plate, and then cooled to room temperature (still in vacuum condition). Monoliths were then grafted with organosilane : 2 monoliths (approximately 0.15 g each) were added into 25 mL of a toluene/organosilane solution of desired concentration. Dynamic vacuum was applied to force the solution into the pores of the monoliths, until effervescence stopped. Monoliths were removed from the grafting solution, and disposed on a grid under toluene-saturated atmosphere for 24 h. They were then washed three times with 25 mL of a toluene/acetone mixture (1:1 v/v). For each washing, wet monoliths were soaked into the washing solvents by applying dynamic vacuum until effervescence stopped and a static vacuum for 2 h. Functionalized monoliths were dried under vacuum at 60 °C for 24 h and then stored in a desiccator at room temperature.

Method D – Controlling water availability

Water availability should be controlled at two levels, in the monolith and in the organosilane solution: monoliths were dried at 120 °C for 24 h under vacuum. After cooling to room temperature (still under vacuum), monoliths were functionalized : 2 monoliths

Experimental

(approximately 0.15 g each) were added into 25 mL of a organosilane solution (in water-saturated toluene) of desired concentration. The saturation water concentration in toluene is 27 mM [168]. Dynamic vacuum was applied to force the solution into the pores of the monoliths, until effervescence stopped. Monoliths were removed from the grafting solution, and disposed on a grid under toluene-saturated atmosphere for 24 h. They were then washed three times with 25 mL of a water-saturated-toluene/acetone mixture (1:1 v/v). For each washing, wet monoliths were soaked into the washing solvents by applying dynamic vacuum until effervescence stopped and a static vacuum for 2 h. Functionalized monoliths were dried under vacuum at 60 °C for 24 h and then stored in a desiccator at room temperature.

APTES concentrations used are : 100, 250, 500, 750 and 1000 mM, that respectively correspond to molar water/APTES ratio of : 0.27, 0.108, 0.054, 0.036 and 0.027.

2.2 Characterizations

2.2.1 Attenuated Total Reflectance – InfraRed Spectroscopy (ATR-FTIR)

Samples were analysed using a Bruker Equinox 55 with a Platinum ATR cell, with a diamond crystal, and Trans DTGS detector. 100 scans were taken for both background and samples, with a resolution of 2 cm⁻¹. ATR correction was applied (number of ATR reflection is 1; angle of incidence is 45°; mean reflection index of sample is 1.5). Monoliths were systematically crushed, unless otherwise stated.

2.2.2 ThermoGravimetric Analysis (TGA)

Samples were dried under vacuum at 105 °C for 24 h. TGA was performed under air flow (50 mL/min) with heating rate of 10 °C/min from 30 °C until 900 °C with a TGA/SDTA-851e Mettler Toledo equipment. Software used for data visualization and treatment was STARE. Differential thermogravimetric data (DTG) is obtained by the ratio between the variation of relative mass loss and the variation of temperature.

2.2.3 Dynamic Light Scattering (DLS)

DLS experiments were performed on a Malvern CGS-3 apparatus equipped with a He-Ne laser with a wavelength of 633 nm. The measurements were performed at a 90° angle. Solutions were analysed without any treatment. Five measurements were taken for

each sample (analysis duration was 30 s for each measurement). The data were analysed using the constrained regularization method for inverting data method, based on inverse-Laplace transformation of the data.

2.2.4 Nitrogen Physisorption (NP)

Adsorption–desorption isotherms of nitrogen were determined at -198 °C using a Micrometrics Tristar 3000 instrument. Samples were degassed at 120 °C under dynamic vacuum for 24 h prior to textural measurements. Specific surface area were determined by the Brunauer-Emmet-Teller Method (BET), in the pressure range between 0.05 and 0.30 (relative pressure).

2.2.5 Scanning Electron Microscopy (SEM)

SEM micrographs were taken with a JEOL 7600F operated at a 15.0 kV voltage. Samples were dried under vacuum at 60 °C for 24 h and then placed on a piece of carbon black tape on an aluminium stub. A chromium sputter coating of 10 nm was carried out under vacuum with a Sputter Metal 208 HR (Cressington).

2.2.6 Thermo-Programmed Calcination (“TPC”)

Crushed samples were analysed using a Catlab-PCS Hiden Analytical equipped with a QGA mass spectrometer. Temperature program was: (i) plateau at 40 °C for 40 minutes, (ii) heating to 800 °C (heating rate 10 °C/min), (iii) plateau at 800 °C for 10 minutes. The air flow rate was 30 mL/min. Detected mass/charge ratios were: 17 (NH₃) and 18 (H₂O), 30 (NO), 44 (CO₂) and 46 (NO₂).

2.2.7 Thermo-Programmed Water Desorption (“TPWD”)

Crushed samples were analysed (as it, without saturation step prior to the analysis) using a Catlab-PCS Hiden Analytical equipped with a QGA mass spectrometer. Temperature program was: (i) plateau at 50 °C for 10 minutes, (ii) heating to 700 °C (heating rate 3 °C/min), (iii) plateau at 700 °C for 10 minutes. The argon flow rate was 30 mL/min. Detected mass/charge ratios were: 17 and 18 (H₂O), 28 (CO), 32 (O₂), 40 (Ar) and 44 (CO₂).

2.2.8 Epoxide titration

Grafted GLYMO is quantified according to Mihailovic *et al* [169]. Titration solution was prepared: 6 mL HBr (48 %_{vol}) is mixed to 250 mL glacial acetic acid. Solution is diluted twice in toluene before titration.

Experimental

Monolith are weighted and crushed, and then placed into an Erlenmeyer flask of 100 mL containing 20 mL toluene, 10 mL glacial acetic acid and 70 μ L violet crystal. Titration solution is added dropwise while Erlenmeyer solution is gently stirred.

Titration ended when solution color goes from purple to emerald green (stable for 2 minutes). Titration is conducted three times for each sample and mean value is presented in the results section. GLYMO content is determined using calibration curve.

2.2.9 Inductively coupled plasma atomic emission spectroscopy (ICP-AES)

Crushed samples were dried at 105 $^{\circ}$ C. Silica-based samples were mineralized with HF/HNO₃ and residues were washed with aqua regia. Agarose-base samples were calcined at 500 $^{\circ}$ C and ashes were mineralized with aqua regia. Metals concentrations were determined using ICAP 6500 (Thermo Schientific).

2.3 Catalytic tests

2.3.1 Enzymatic activities determination (in batch mode)

2.3.1.1 Transaminases

A model reaction was used to assess catalytic activity of transaminases: the transamination of pyruvate with racemic BMBA, to produce BAP and D-alanine. As the enzyme only accepts the R-enantiomer of BMBA as a substrate, this reaction is a kinetic resolution (Figure E - 1). Typical conditions were 30 $^{\circ}$ C, phosphate buffer 0.1 M pH 8, pyridoxal phosphate 2.02 mM, sodium pyruvate 10 mM, racemic BMBA 10 mM, DMSO 5 %. Batch reactions were carried out in 5 mL round bottom glass flasks under moderate magnetic stirring.

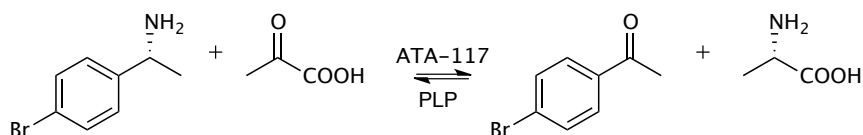


Figure E - 1 – Kinetic resolution. As the transaminase ATA-117 is R-selective, it only accepts the R-BMBA as a substrate, leaving the S-BMBA unreacted.

Transamination reaction conversion was determined on 100 μ L sample taken from the reaction medium. 10 μ L of sodium hydroxide

(2 M) was added and the mixture was vortexed for 1 second. 500 μ L of dichloromethane was then added to the aqueous phase and vortexed for 10 seconds to allow extraction of BAP and BMBA into the organic phase. Extraction step was repeated twice and the organic phase was collected and analysed by gas chromatography (analysis performed on a Bruker Scion 456-GC with a WCOT fused silica BR-5 column (30 m x 0.32 mm ID x 1.0 μ m) and helium as carrier gas (25 mL/min), oven temperature at 150 $^{\circ}$ C, split ratio of 20, injector temperature at 250 $^{\circ}$ C, flame ionization detector temperature at 300 $^{\circ}$ C (air flow 300 mL/min, H₂ flow 30 mL/min)). The yield is defined as the proportion of rac-BMBA converted into 4'-bromoacetophenone (BAP; in %). The maximum yield for the kinetic resolution is thus 50%.

Enantiomeric excess was determined using Chiral High Performance Liquid Chromatography (Chiral-HPLC). R and S-enantiomers of bromo- α -methylbenzylamine were detected and quantified by normal phase HPLC (Hitachi-koki, Chiyoda, Japan) at 252 nm using a Merck-Hitachi LaChrom L-7000 HPLC and a Chiralpak AD-H (250 mm x 4.6 mm) column with a flow rate of 1 mL/min (90% isohexane/10% 2-propanol/0.1% diethylamine) for 10 min. The retention times of the S- and R-enantiomers were 4.32 and 5.42 min, respectively. Specific rotation of the BMBA product was established by comparison to known standards.

2.3.1.2 Yeast Alcohol Dehydrogenase (YADH), Lactate Dehydrogenase (LDH) and Glucose Dehydrogenase (GDH)

Dehydrogenases (LDH, YADH and GDH) activities were determined by measuring the NADH concentration over time (Figure E - 2, Figure E - 3 and Figure E - 4). These reactions were performed at 30 $^{\circ}$ C in phosphate buffer (100 mM, pH 8).

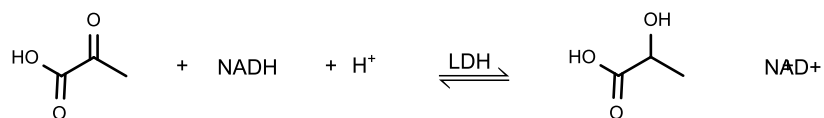


Figure E - 2 – The reduction of pyruvic acid by NADH catalysed by the LDH. The reaction produces lactic acid as a by-product.

Experimental

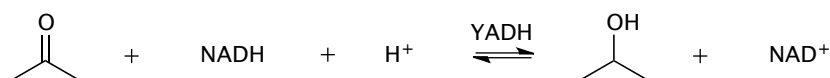


Figure E - 3 – The reduction of acetone by NADH catalysed by the YADH. The reaction produces isopropyl alcohol as a by-product.

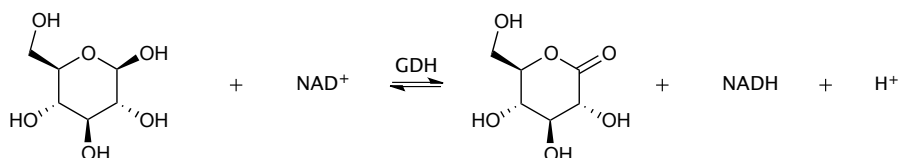


Figure E - 4 – The oxidation of glucose by NAD⁺. It allows to recover the NADH and produce δ -gluconolactone as a by-product. This reaction is catalysed by GDH enzyme.

NADH absorbance was measured at 340 nm with a spectrophotometer Vis ThermoScientific Genesys 10S-Vis. Samples were diluted in phosphate buffer in order to not exceed 1 mM NADH in the cell: typically, 400 μ L of reaction medium were added to 600 μ L of phosphate buffer.

2.3.1.3 Tyrosine Ammonia-Lyase (TAL)

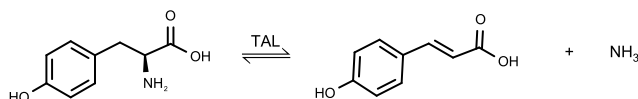


Figure E - 5 – Tyrosine deamination. It leads to the production of coumaric acid and ammonia. This reaction is catalysed by TAL.

TAL activity was assessed using model reaction (Tyrosine deamination leading to the production of coumaric acid and ammonia; Figure E - 5) in UV range using a SpectraMax M5 spectrometer from MolecularDevices, with the SoftMax Pro software. Quartz or polymethylmethacrylate (PMMA) cuvettes were used. Spectra of substrates and reactants solution (in buffer) were first recorded to determine the maximum of absorbance of such molecules. Then, calibration curves were achieved at fixed wavelength for tyrosine, tyrosine methyl ester and tyrosine ethyl ester (respectively at 290 nm and 310 nm for both esters). Buffer solution was used as a reference (blank).

Enzyme kinetics was recorded at specific wavelength (290 or 310 nm) starting from the addition of enzyme followed by a quick

inversion mixing. Reaction conditions were typically: 50 μ M tyrosine in buffer (150 mM NaCl, 50 mM TRIS-HCl, pH 7.5). Absorbance was measured every 2 seconds over a period of 5 minutes. Cuvettes were maintained at 30 °C inside the spectrometer.

2.3.1.4 Cascade systems

Cascade reactions in batch mode were performed in 5 mL round bottom flask, under moderate stirring. Specific conditions are detailed in Annexe 0. BMBA, BAP and FPA were extracted, detected and quantified in gas chromatography, using the same device and parameters as for Experimental, Section 2.3.1.1.

2.3.2 Enzymatic flow reactions

2.3.2.1 Flow reactor

A monolith was inserted into a heat-shrinkable PTFE tube, which perfectly fits the monolith shape when heated (Figure E - 6) preventing preferential flows along monolith sides (*vide infra*). PTFE tube was heated using a heat gun, maintained at a distance of at least 10 cm from the tube. Heat-shrinkable PTFE tube was also shrunk around stainless steel male connectors (surrounded by PTFE tape to ensure sealing), themselves connected to PTFE pipes. Three identical copies of this flow device were built and each of them was connected to a PP Terumo syringe. Three syringe-pumps were used to deliver a controlled liquid flow. Monolithic reactors were placed in a thermo-regulated water bath (Figure E - 7).

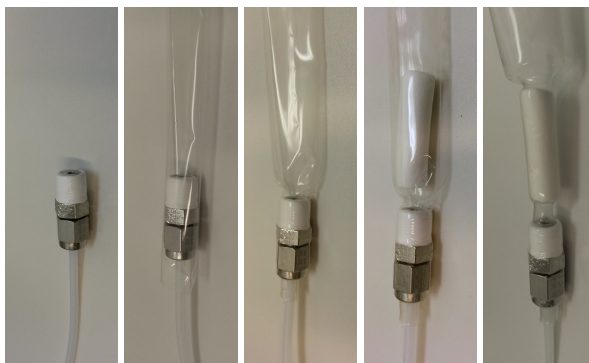


Figure E - 6 – Flow reactor construction. Heat-shrinkable PTFE tube shrunk around Swagelok connectors (covered by PTFE seal tape) and Si(HIPE) monolith (from left to right).

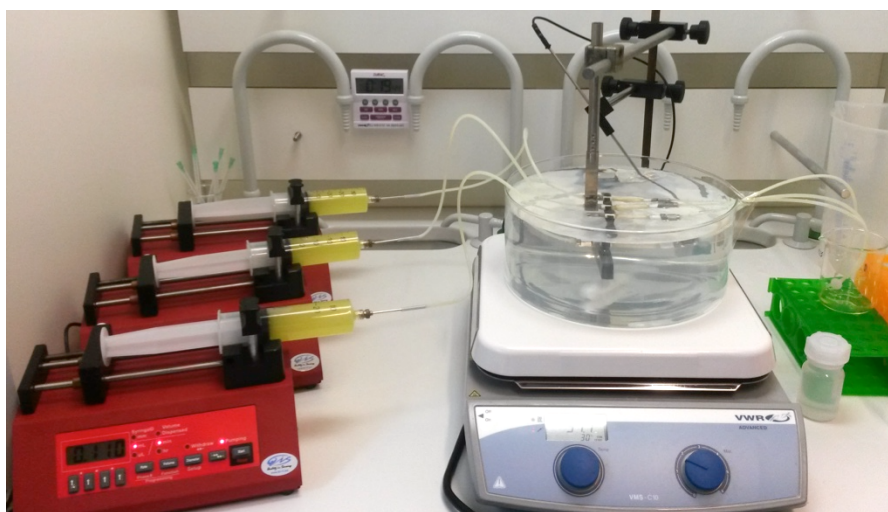


Figure E - 7 – Experimental setup. Monolithic reactors are connected to syringe-pumps and immersed in a water bath.

2.3.2.2 Flow process

Three different flow enzymatic processes have been conducted in this work : (i) the flow transamination in the kinetic resolution mode, (ii) the flow transamination in the asymmetric synthesis mode, and (iii) the flow tyrosine deamination. In each cases, porous volume was estimated by weighing monolith before and after reaction (reaction medium density was measured at 1.0024).

i. Transamination (kinetic resolution)

- Transaminase immobilization (through simple adsorption, adsorption enhanced by electrostatic or hydrophobic interactions, and covalent grafting)

Each reactor was impregnated with 50 mL of desired transaminase concentration solution in buffer (potassium phosphate buffer 0.1 M pH 8, pyridoxal phosphate 2.02 mM, sodium pyruvate 10 mM) with a flow rate of 1.4 mL/min, with the water bath maintained at 4 °C with ice packs or heated at 30 °C. Then, the reactor was washed at 30 °C with 50 mL buffer, with a flow rate of 1.4 mL/min, until no enzyme is detected in the outflow. Practically, this was achieved after using approximately 30 mL of buffer solution (see Chapter I). In some cases (covalent grafting), 50 mL of a 1 % glutaraldehyde aqueous solution was passed through the reactor (flow rate of 1.4 mL/min) at 4

°C with ice packs or heated at 30 °C before enzyme impregnation (then, the catalyst is denoted « -30d »).

- Transaminase immobilization (through His-tag)

All the steps are processed at 30 °C (Figure E - 8). Each reactor (GLYMO-grafted monoliths) was impregnated with 15 mL of Lys-NTA (0.01M in water) with a flow rate of 1.4 mL/min. Then the reactor is washed with 15 mL water (1.4 mL/min). Nickel solution (0.1 M NiCl_2 in water) is then injected in the reactor (15 mL at a 1.4 mL/min flow rate) prior to a washing with water (15 mL, 1.4 mL/min). Then, transaminase solution in buffer (potassium phosphate buffer 0.1 M pH 8, pyridoxal phosphate 2.02 mM, sodium pyruvate 10 mM) is injected with a flow rate of 1.4 mL/min. Then, the reactor was washed at 30 °C with 50 mL buffer, with a flow rate of 1.4 mL/min, until no enzyme is detected in the outflow.

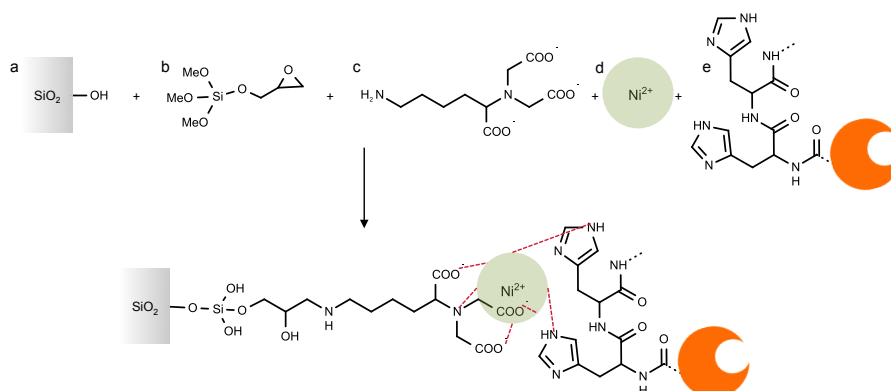


Figure E - 8 – Immobilization through His-tag. (a) Silica is grafted with (b) GLYMO. (c) Lys-NTA is reacted with epoxide function. (d) Nickel ions are complexed by carboxylic functions and nitrogen atom. (e) His-tagged enzyme is immobilized on this NTA:Ni complex, through complexation (with nitrogen located on the imidazole rings).

- Flow transamination reaction

The model reaction (see Figure E - 1) was used to assess catalytic activity of ATA-117. Reaction medium (same conditions as in batch reactions) was loaded in syringe and injected into the reactor with a flow rate of 0.11 mL/min. Catalytic activity was determined by collecting the outflows and measuring BMBA consumption and BAP production in gas chromatography. During enzymatic reaction, actual flow rates were continuously monitored allowing an accurate determination of contact time.

Experimental

ii. Transamination (asymmetric synthesis)

- Enzymes immobilization

50 mL of a 1 % glutaraldehyde aqueous solution was passed through the reactor (flow rate of 1.4 mL/min) at 30 °C (catalysts name contains « -30d ») before enzyme impregnation. Reactors were then impregnated with 50 mL of enzyme solution containing transaminase, LDH and GDH in various concentrations in buffer (potassium phosphate buffer 0.1 M pH 8, pyridoxal phosphate 2.02 mM) with a flow rate of 1.4 mL/min, with the water bath heated at 30 °C. Then, the reactor was washed at 30 °C with 50 mL buffer, with a flow rate of 1.4 mL/min.

- Flow transamination reaction

The cascade reaction (see Chapter V) was used to assess catalytic activity in the asymmetric synthesis mode. Reaction medium (BAP 5 mM, Alanine 50 mM, PLP 0.5 g/L, DMSO 5 %, NADH 100 µM, NAD 100 µM, d-Glucose 50 mM, in phosphate buffer pH 8, 100 mM) was loaded in syringe and injected into the reactor with a flow rate of 0.11 mL/min. Catalytic activity was determined by collecting the outflows and measuring BAP consumption and BMBA production in gas chromatography.

iii. Tyrosine deamination

- TAL immobilization

50 mL of a 1 % glutaraldehyde aqueous solution was passed through the reactor (flow rate of 1.4 mL/min) at 30 °C before enzyme impregnation. Reactors were then impregnated with 50 mL of TAL solution (typically 3 mg) in buffer (50 mM TRIS-HCl, pH 7.5, 150 mM NaCl) with a flow rate of 1.4 mL/min, with the water bath heated at 30 °C. Then, the reactor was washed at 30 °C with 50 mL buffer, with a flow rate of 1.4 mL/min.

- Flow tyrosine deamination

The tyrosine deamination reaction (see Figure E - 5) was used to assess catalytic activity of TAL. Reaction medium (50 µM Tyrosine in buffer (150 mM NaCl, 59 mM TRIS-HCl, pH 7.5)) was loaded in syringe and injected into the reactor with a flow rate of 0.11 mL/min. Catalytic activity was determined by collecting the outflows and measuring absorbance in UV spectrometry.

2.3.2.3 Loading evaluation

Enzymatic concentrations were mostly assessed by a modified Bradford method [170,171] (see Annexe 2), using the Bovine Serum Albumin protein as a standard. 500 μ L of sample were added to 1500 μ L of Bradford reagent. After 10 minutes incubation at room temperature, absorbances were read at 595 and 470 nm with a spectrophotometer ThermoScientific Genesys 10S-Vis.

Some transaminase quantifications were performed using residual activities: 400 μ L of sample containing transaminases were added to a 2 mL vial containing 10 mM BMBA, 5 % DMSO, 0.5 g/L PLP, 10 mM sodium pyruvate, phosphate buffer (pH 8); and incubating for 3 hours at 30 °C. BAP concentration of such vials were assessed using extraction and GC methods of Experimental, Section 2.3.1.1. Calibration curve was performed plotting a range of transaminase concentration in sample (from 0 to 0.3 g/L) *versus* the BAP concentration after 3 hours.

Enzyme loading in flow biocatalysts was determined by assessing the enzyme concentration in the inflows and the outflows.

Chapter I – Immobilization of transaminases on silica monolith for flow transaminations – Proof of concept

1 Introduction

This first chapter was intended to demonstrate the feasibility of the system: the use of transaminases immobilized on Si(HIPE) monolith for flow enantioselective transaminations. To that end, ATA-117 transaminase has been thoroughly studied in batch mode for the determination of optimal conditions (in preparation, storage and working conditions). Si(HIPE) materials suitable for flow reactions have been produced and a flow reactor has been designed. Materials have been characterized by infrared spectroscopy (surface chemistry), scanning electron microscopy (morphology), nitrogen physisorption (textural properties), thermogravimetric analysis (organic content). Then flow reactions have been processed in the way of kinetic resolution (to get rid of non-favoured amination), using transaminases immobilized on Si(HIPE) as biocatalysts suitable for flow chemistry. In the perspective of an industrial application, we verify that the solid biocatalysts are active, stable over time, enantioselective, and do not suffer from diffusional limitations.

The results presented in this chapter are published in [1]:

Ludivine van den Biggelaar, Patrice Soumillon, Damien P. Debecker, *Catalysts*, Enantioselective Transamination in Continuous Flow Mode with Transaminase Immobilized in a Macrocellular Silica Monolith, **7** (2017) 54.

2 Results and discussion

2.1 ATA-117 activity in batch mode

Transaminase ATA-117 has been studied in batch mode using the model reaction below (Figure 1 - 1), prior to immobilization, in order to determine its optimal stability conditions (preparation conditions, pH, storage, see Annexe 1).

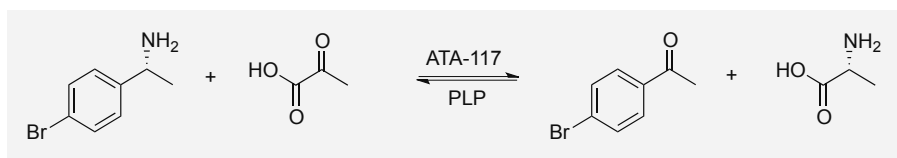


Figure 1 - 1 – Model reaction for assessing ATA-117 activity. R-methylbenzylamine (R-BMBA) reacts with pyruvic acid in the presence of ATA-117 and PLP, to form bromo-acetophenone (BAP) and alanine. In practice, a racemic mixture of BMBA is used. This reaction is a kinetic resolution.

Then, kinetic parameters have been measured: in the best preparation and pH conditions, the transaminase ATA-117 converts 50 % of R-BMBA in 30 minutes (Figure 1 - 2). This activity corresponds to a global rate constant, k_{obs} , of 0.023 min^{-1} (see detailed calculation in Annexe 1.1).

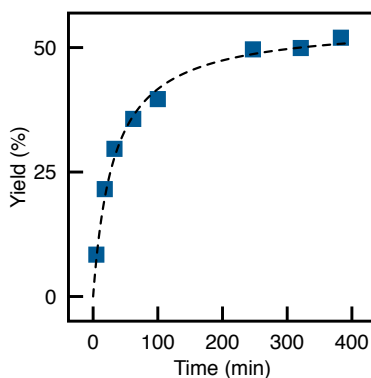


Figure 1 - 2 – Activity of ATA-117 in batch mode. Experimental conditions: ATA-117 (0.16 g/L); rac-BMBA (10 mM), pyruvic acid (10 mM), PLP (0.5 g/L), pH 8, 30 °C, reactor volume 5 mL. The yield, *i.e.* the conversion of racemic BMBA into BAP, increases over time to reach a plateau at 50 %. Dotted line acts as a guide to the eye.

This experiment was performed with 4.4 μM transaminase (0.16 g/L, theoretical concentration, if assuming ATA-117 stock is pure), allowing to calculate the catalytic efficiency, *i.e.* the ratio of the catalytic rate constant (k_{CAT}) over the Michaelis-Menten constant (K_m), which is about $5.2 \cdot 10^3 \text{ M}^{-1} \cdot \text{min}^{-1}$ (see Annexe 1). This ratio is quite low as compared to other transaminases that reach $10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$ [172]. This could be explained by the fact that ATA-117 activity is assessed by a model reaction involving a non-natural substrate (BMBA) instead of its natural one (amino acid). Also, the ATA-117 concentration may have been overestimated due to a low purity in the ATA-117 stock (see Chapter IV). In addition, this concentration has been chosen in order to have a good compromise between rapid conversion and ease in initial rate measurements (several points on the area where the yield increases linearly with time). Besides, we must note that we do not know the exact purity of ATA-117 stock. Then, when discussing ATA-117 mass, the unit “g” is expressed as the mass of lyophilized powder (and then, taking account of impurities in that powder).

Enantioselectivity of ATA-117 was checked by chiral-HPLC (Figure 1 - 3). ATA-117 is R-selective as only the R-enantiomer of BMBA is consumed over time, while S-BMBA concentration remains constant.

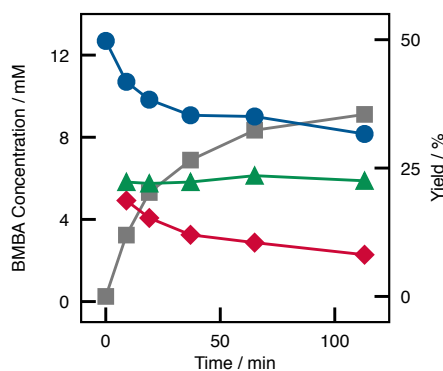


Figure 1 - 3 – Batch transamination with ATA-117. Experimental conditions: ATA-117 (0.16 g/L); rac-BMBA (10 mM), pyruvic acid (10 mM), PLP (0.5 g/L), pH 8, 30 °C, reactor volume 5 mL. (●) Total BMBA concentration follows (◆) R-BMBA concentration while (▲) S-BMBA concentration remains stable over time, indicating that only R-BMBA is converted by ATA-117 transaminase. Total BMBA concentration is assessed by GC, enantiomeric excess is assessed by chiral-HPLC. (■) Yield is assessed through BAP concentration (GC).

2.2 Si(HIPE) monolith

2.2.1 Synthesis and characterization

Synthesis of Si(HIPE) support is described in Experimental, Section 2.1.2.1. Briefly, an emulsion is produced with a high proportion of dodecane as the dispersed phase and an acidic aqueous continuous phase containing tetraethyl orthosilicate. Hydrolysis and condensation reactions occur around oil droplets and lead to the formation of a macrocellular silica scaffold. After the removal of the oil phase, a self-standing monolith is obtained, featuring high specific surface area ($820 \text{ m}^2 \cdot \text{g}^{-1}$, Figure 1 - 4a, and see Section Experimental, 2.2.4), mainly explained by microporosity due to silica lattice molecular structure, and large void fraction. Monoliths geometry has been standardized by always casting the emulsion in containers of identical shapes. We obtained monoliths with $\sim 3.5 \text{ cm}$ in height and $\sim 0.9 \text{ cm}$ in diameter, with same weight (0.15 g ; Figure 1 - 4b). Monoliths density is $0.0628 \text{ g} \cdot \text{cm}^{-3}$, porosity reaches $\sim 95 \%$ (if bulk density is assumed to be $1.56 \text{ g} \cdot \text{cm}^{-3}$ [123]). Si(HIPE) consists of empty interconnected spherical silica shells whose shape is a reminiscence of the dodecane droplets in the oil-in-water emulsion (Figure 1 - 4c). Polycondensation of TEOS into silica starts from the organic/aqueous interface towards the rest of aqueous phase [123]. The diameter of the macrocellular shells is highly variable (from $\sim 1 \mu\text{m}$ to $300 \mu\text{m}$).

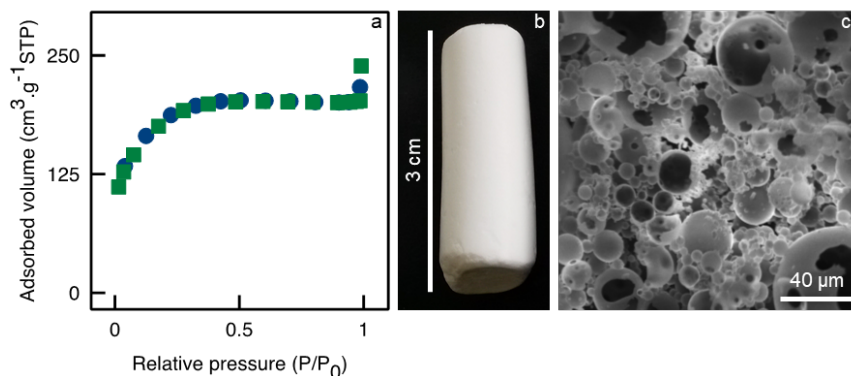


Figure 1 - 4 – Textural properties and morphology of Si(HIPE). Nitrogen (■) adsorption and (●) desorption isotherms for Si(HIPE). STP stands for Standard Temperature and Pressure. (b) Cylindrical Si(HIPE) monolith of standard geometry. (c) SEM micrograph of Si(HIPE) monolith showing the interconnected silica spheres.

ATR-FTIR analysis of Si(HIPE) showed all the features typically found for silica (Figure 1 - 5): isolated silanols (3750 cm^{-1} [173]), H-

bounded water band (broad signal in the $3460\text{--}3000\text{ cm}^{-1}$ range [174]), water (1640 cm^{-1} [174]) and SiO_2 lattice vibrations band ($1200\text{--}1000\text{ cm}^{-1}$ [175]).

Thermogravimetric analysis of Si(HIPE) showed an important mass loss below $150\text{ }^\circ\text{C}$ assigned to surface water elimination [176], and to water located in the biggest pores [136]. Over this key-temperature, the water located in the small pores (micropores) is gradually evacuated. At the same time, silanols condensation into siloxane bridges occurred (producing water as a by-product) [136]. Also, organic contamination is decomposed above $150\text{ }^\circ\text{C}$. Despite the washing and calcination steps (see Experimental, Section 2.1.2.1) that would lead to a total removal of organic matter (dodecane, solvent and surfactant), organic matter is still observed in Si(HIPE). This organic matter is assigned to external contamination (from handling in air, between the moment the monolith is withdraw from the oven and the moment it is characterized in TGA). However, this contamination is very weak ($< 3\%$ of Si(HIPE) mass).

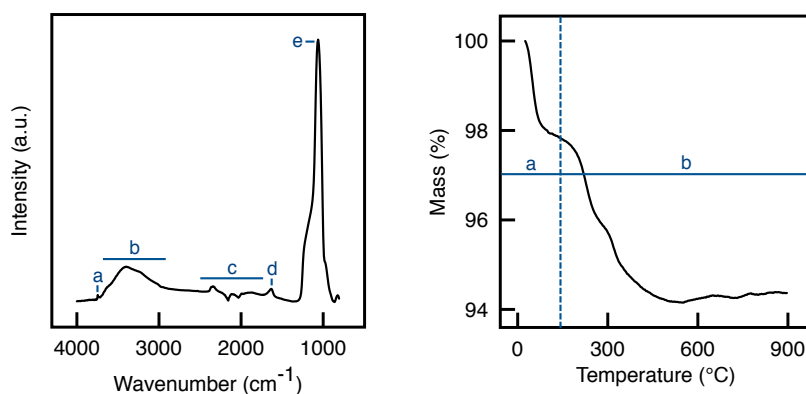


Figure 1 - 5 – ATR-FTIR spectrum and TGA of Si(HIPE). (Left) Si(HIPE) monoliths exhibit all the features typically found for silica materials: (a) isolated silanols, (b) H-bounded water, (d) water, (e) SiO_2 lattice vibrations. The bands located in the (c) zone are artefacts associated to a poor diamond crystal transmittance area [177]. (Right) TGA of Si(HIPE) showed (a) an important mass loss due to surface water elimination (and water located in the macro- and mesopores). (b) Over $150\text{ }^\circ\text{C}$, water from micropores is gradually lost. In the same time, silanols condensation into siloxane bridges occurred, and residual organic matter is decomposed.

2.2.2 Design of flow reactor suitable for silica monolith as fixed bed

During the synthesis process, Si(HIPE) monoliths undergo an important macroscopic shrinkage attributed to the densification of the silica network. Thus, the final diameter of silica monoliths was not the

same as the diameter of the mold. Consequently, we had to find a container that perfectly fits the shape of the calcined monoliths. But, the prediction of the final diameter of the monoliths was not straightforward (due to small batch-to-batch variations in the shrinkage). Consequently, a heat-shrinkable material was implemented to match the shapes of the monolith. This plastic material shrinks when heated, without damaging the monolith (see Experimental, Section 2.3.2.1). A polyolefin heat-shrinkable membrane was initially implemented. However, this material adsorbed a significant amount of rac-BMBA, making this material unsuitable for flow reactions. A polytetrafluoroethylene (PTFE) heat-shrinkable membrane was shown to be inert towards flow components and was finally implemented.

A peristaltic pump connected with silicone flexible tubes was initially used as a pumping device. However, the silicone material showed an important adsorption of flow components (rac-BMBA, enzymes), making the use of peristaltic pump inadequate. A syringe pump connected to PTFE tubes and PTFE syringes was finally chosen as an inert pumping device (see Experimental, Section 2.3.2.1).

Prior to flow biocatalysis experiments, the buffer (which contains PLP, a yellow compound) was injected into a Si(HIPE) monolith itself placed in the continuous flow set-up. The flow was stopped before the yellow coloration reached the end of the monolith. The monolith was then recovered and cut lengthwise in order to observe the flow profile (Figure 1 - 6). The boundary between yellow zone (filled with buffer) and the white zone (not yet reached by the buffer) is straight. Also, to the eye, the yellow zone is uniform in colour intensity. This indicates that the liquid follows an ideal plug flow regime into Si(HIPE). Therefore, it is assumed that product concentration into the reactor depends only on the relative position on the axis parallel to the flow (rather than on the radial position).

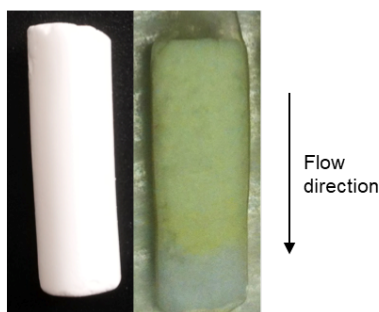


Figure 1 - 6 – Flow profile in Si(HIPE) monolith. (Left) Si(HIPE) monolith. (Right) Si(HIPE) monolith removed from the set-up during impregnation of coloured buffer and cut lengthwise.

2.3 Enzyme immobilization on Si(HIPE) monoliths

The transaminase ATA-117 was immobilized on Si(HIPE) by three methods (Figure 1 - 7): (a) simple adsorption, (b) adsorption facilitated by electrostatic interactions (AFEI), (c) covalent grafting. For the simple adsorption, the enzyme solution (0.2 g/L, pH 8) was simply allowed to flow through the bare silica monolith (in the flow set-up, see Experimental, Section 2.3.2). The monolith is then washed with the buffer solution (catalyst denoted “TA-Si(HIPE)”, where TA stands for transaminase). The washing buffer is composed of phosphate buffer (pH 8, 100 mM) and contains PLP (0.5 g/L) and sodium pyruvate (10 mM).

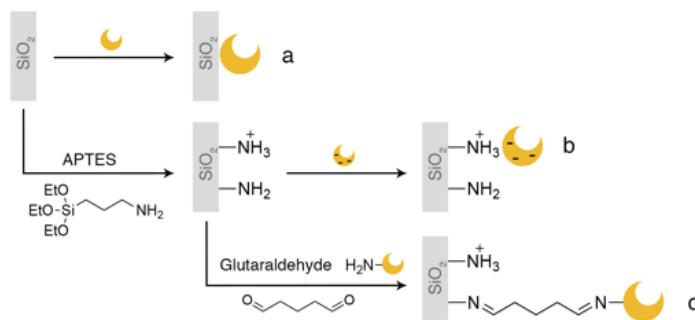


Figure 1 - 7 – Immobilization methods. (a) Simple adsorption, (b) adsorption assisted by electrostatic interactions and (c) covalent grafting.

The external surface of ATA-117 is negatively charged at the optimal working pH (pH = 8; see Introduction, Section 2.2.2). Thus, when using AFEI method, the silica monolith was functionalized by APTES (with the Method A, see Experimental, Section 2.1.2.3) to bring amino functions that are positively charged at pH = 8; and the

enzyme adsorption was reinforced by attractive electrostatic interactions. The initial amount of APTES introduced in the preparation of the support was varied and the catalysts are denoted "TA-Ax_Y", where A stand for APTES and x is the APTES concentration used in the functionalization step, Y is the functionalization method involved (see Experimental, Section 2.1.2.3).

ATA-117 external surface also exhibits six lysine residues (see Introduction, Section 2.2.2). In the covalent grafting method, glutaraldehyde was used as a coupling agent [105,178] to promote the covalent grafting between the amino functions on the APTES-functionalized Si(HIPE) and the lysine residues of the enzyme (catalysts denoted "TA-Ax_Y-GA" where GA stand for glutaraldehyde).

For AFEI and covalent grafting methods, monoliths were functionalized with APTES, using the "method A", adapted from Ungureanu *et al* [157].

The efficiency of APTES functionalization was verified by attenuated total reflectance infrared spectroscopy (ATR-FTIR). Signals related to the amino functions are difficult to identify: N-H vibration at 1610 cm⁻¹ is overlapped by water band (1600-1640 cm⁻¹) [175]; and C-N vibration at 2259 cm⁻¹ is on a poor diamond crystal transmittance area [174]. However, an increase in intensity of absorbance band assigned to C-H stretching [140,175] is clearly observed (from 2885 to 2974 cm⁻¹) when comparing the APTES-functionalized support with the pristine one (Figure 1 - 8). Expectedly, the intensity of this band increases with the APTES concentrations used for functionalization. Compared to the IR spectrum of pure APTES, the spectra of APTES-functionalized Si(HIPE) samples show a more intense band for CH₂ vibrations (2855 and 2926 cm⁻¹) and less intense band for CH₃ stretching vibrations (2885 and 2974 cm⁻¹) [140,175]. This indicates that ethoxy groups of APTES are eliminated during functionalization, consistent with the silanization process where ethoxy groups react with the silanol groups of the support (see Introduction, Section 2.3.4).

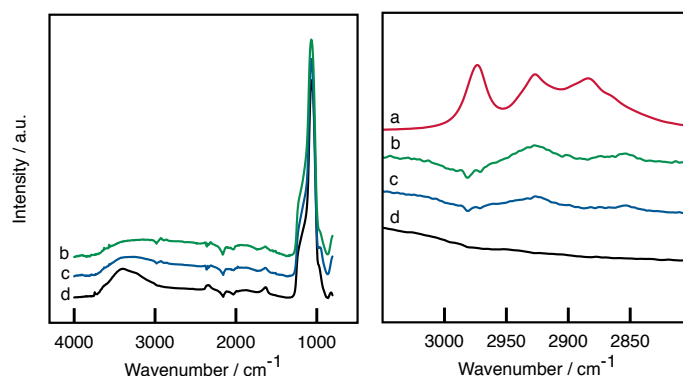


Figure 1 - 8 – ATR-FTIR spectra of grafted monoliths. (Left) Global spectra, (right) focus around 2900 cm^{-1} . (a) Pure APTES, (b) A50_A, (c) A10_A and (d) Si(HIPE). An increase in C-H stretching band absorbance (around 2900 cm^{-1}) suggests a higher amount of aminopropyl groups grafted to monolith surface for higher APTES concentrations.

Thermogravimetric analyses confirmed the presence of additional organic species in the APTES-functionalized samples (Figure 1 - 9). A relatively well-defined weight loss is observed between 300 and $600\text{ }^{\circ}\text{C}$ with the functionalized samples (and not with the pristine Si(HIPE)). It is thus assigned to aminopropyl moiety decomposition [176,179]. The weight loss is larger when the APTES concentration used to functionalize the silica monolith is higher.

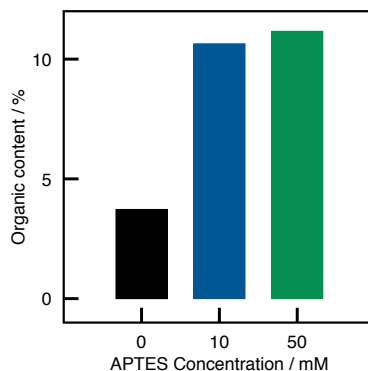


Figure 1 - 9 – TGA of Si(HIPE), A10_A and A50_A samples.

After APTES functionalization, SEM micrographs show that the morphology of the samples is preserved (Figure 1 - 10). Indeed, hollow, spherical, and interconnected silica shells appear undamaged.

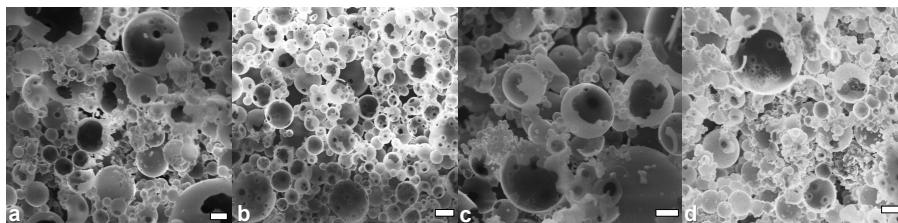


Figure 1 - 10 – SEM micrographs. (a) Si(HIPE), (b) A10_A, (c) A50_A and (d) TA-A50_A-GA (after the reaction, TA immobilized through covalent grafting – with glutaraldehyde). Material is made of interconnected silica hollow spheres. Functionalization process and enzymatic reaction do not affect sample morphology, as spherical shells appear undamaged. Scale bar is 10 μ m.

In principle, the enzyme loading in each monolith can be evaluated by difference, by a simple mass balance between the solution used to load the monolith and the recovered solution, including the washing solution (see Experimental, Section 2.3.2.3). Unfortunately, these measurements suffered from high variability and discrepancies, which prevent us from providing the precise enzyme loading for each monolith (as discussed in Annexe 2). As a general trend however, TA-Si(HIPE) and TA-Ax_A with low APTES content showed low enzyme loading values, certainly below 0.5 mg of enzyme in the monolith. For the TA-Ax_A-GA catalysts with high APTES content, the loading is clearly higher and it is possible to provide an estimation of the enzyme loading. In TA-A50_A-GA – the most active catalyst, *vide infra* – enzyme loading was estimated at 1.2 mg (mean of significant protein determination results, *i.e.* the positive values).

2.4 Transaminations in flow mode

The kinetic resolution of racemic bromo- α -methylbenzylamine (rac-BMBA) into 4'-bromoacetophenone (BAP) was studied as a model transamination reaction to evaluate the performance of the solid biocatalysts (Figure 1 - 11). The thermodynamic equilibrium for this reaction is highly favourable and a conversion of ~99 % is expected at equilibrium [180,181]. However, without any catalyst, this conversion is very slow (Figure 1 - 12). In the presence of ATA-117, a *R*-selective transaminase, *R*-BMBA conversion into BAP is strongly catalysed. In this work, the yield is defined as the proportion of rac-BMBA converted to 4'-bromoacetophenone (BAP; in %). The maximum yield for the kinetic resolution is thus 50 %.

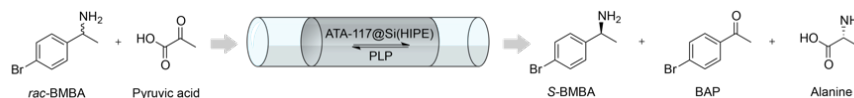


Figure 1 - 11 – Flow kinetic resolution of *rac*-BMBA by ATA-117 transaminases immobilized on Si(HIPE) monolith. As ATA-117 is *R*-selective, it only accepts the *R*-BMBA as a substrate, leaving the *S*-BMBA unreacted. PLP stands for pyridoxal phosphate, the transaminase cofactor.

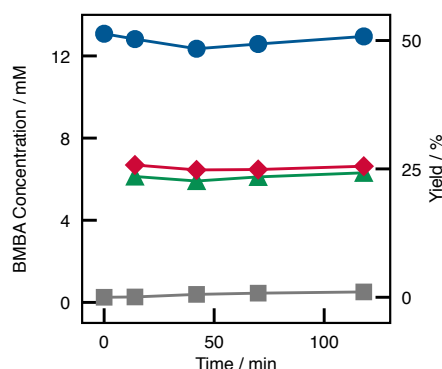


Figure 1 - 12 – Batch reaction without ATA-117. Experimental conditions: *rac*-BMBA (10 mM), pyruvic acid (10 mM), PLP (0.5 g/L). (●) Total BMBA concentration and (■) yields are relatively stable over time. Ratio between (◆) *S* and (▲) *R* enantiomer remains constant. This suggests that the non-enzymatic slow reaction does not favour the consumption of any of the enantiomers. While ATA-117 is *R*-selective the slow non-enzymatic conversion is thus not enantioselective as no BMBA enantiomer is preferentially consumed.

The pristine Si(HIPE) monolith was initially tested as a support for the transaminase (sample TA-Si(HIPE)). The yield was moderate, decreased in the first hour of reaction, and stabilized around 1.1 % (Figure 1 - 13). Initial deactivation may tentatively be attributed to a partial leaching of the enzyme out of the monolith. Functionalization of the surface with APTES is expected to modify the surface charge of the silica support and to favour the electrostatic adsorption of the enzyme on the support. Indeed, higher conversion was obtained with TA-A10_A. A true covalent anchoring of the enzyme should be obtained under the action of glutaraldehyde used as coupling agent between the amine functions incorporated on the surface of the support and the amine functions of the enzyme. Pre-activation with glutaraldehyde (TA-A10_A-GA) seems to allow reaching a stable yield of 2.1%, although the effect is not significantly better than for TA-A10_A. We must note that a very slow conversion occurs under the sole effect of cofactor PLP (without enzyme; Figure 1 - 14). Indeed,

the cofactor is able to slowly catalyse the non-enzymatic (and non-enantioselective) transamination.

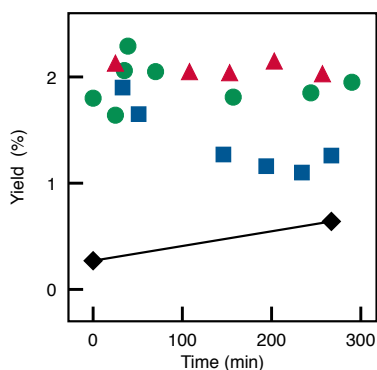


Figure 1 - 13 – APTES and glutaraldehyde pre-activation effects on biocatalysts efficiency in flow. Experimental conditions: ATA-117 (10 mg in impregnation solution); rac-BMBA (10 mM), pyruvic acid (10 mM), PLP (0.5 g/L). Carriers: (■) Si(HIPE), (●) TA-A10A, (▲) TA-A10A-GA. (◆) Reagent solution.

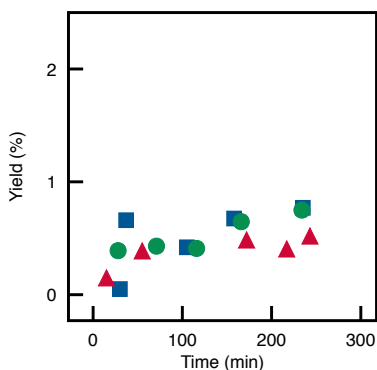


Figure 1 - 14 – Non-enzymatic conversion (no enzyme). Experimental conditions: rac-BMBA (10 mM), pyruvic acid (10 mM), PLP (0.5 g/L). It is noticed that a slow non-enzymatic and non-enantioselective conversion occurred in the reactant solution under the sole effect of PLP: only a low conversion occurred for (●) batch and flow non enzymatic reactions performed in the same conditions. No additional conversion is observed when reactant solution passed through (■) Si(HIPE) or (▲) A50A monolith, suggesting that silica and APTES show no catalytic activity.

In an attempt to further increase the reaction yield, the APTES concentration in the functionalization solution was raised to 20 and 50 mM, keeping all other parameters constant. The yield increased when the concentration of APTES used for functionalization increased (Figure 1 - 15), expectedly suggesting that a higher density of grafted

APTES on the monolith surface is leading to a higher enzymatic loading. The highest BAP yield was obtained for TA-A50_A-GA sample (6.2 %). The experiment has been carried out 3 times and showed a good repeatability (Relative standard deviation (RSD) was 9 %). As flow experiments are time-consuming, they are not systematically repeated. We gave trust to RSD obtained with TA-A50_A-GA sample, and we hypothesized that similar RSD are obtained for similar biocatalysts.

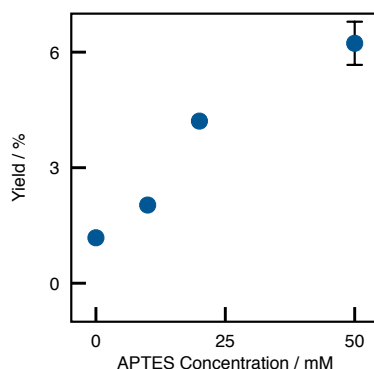


Figure 1 - 15 – Effect of APTES concentration in the grafting solution on biocatalysts efficiency. Experimental conditions: ATA-117 (10 mg in impregnation solution); rac-BMBA (10 mM), pyruvic acid (10 mM), PLP (0.5 g/L). Increasing APTES concentration allows enhancing the yield. Yield values are the averaged yield measured after 180 min time on stream (when the yield appeared to stabilize).

This level of conversion was achieved with a 10 min contact time. If the flow rate and the size of the monolith are varied in parallel while keeping the contact time constant, the yield remains at the same level (Figure 1 - 16). This confirms that the activity measurement is not biased by diffusional limitations.

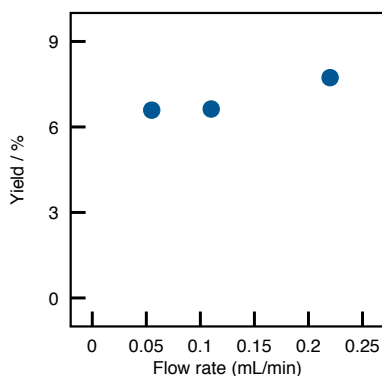


Figure 1 - 16 – Dependence of conversion on flow rate, for TA-A50_A-GA..
Experimental conditions: ATA-117 (10 mg in impregnation solution); rac-BMBA (10 mM), pyruvic acid (10 mM), PLP (0.5 g/L). The contact time is constant by adjusting the flow rate on the bed size. The yield was constant suggesting that no diffusional limitation occurred for the flow rates chosen.

Also, the level of conversion is far enough from the thermodynamic equilibrium to be considered as a measurement of the initial activity. Indeed, conversion is roughly divided by two or multiplied by two when the reaction is run with half a monolith or with two monoliths respectively (Figure 1 - 17).

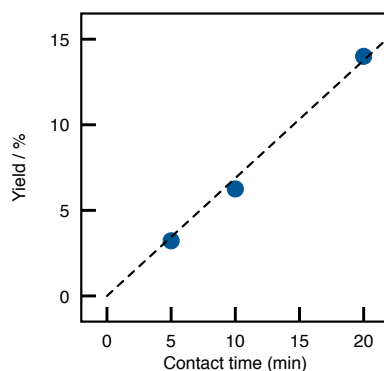


Figure 1 - 17 – Dependence of conversion on contact time, for TA-A50_A-GA.
Experimental conditions: ATA-117 (10 mg in impregnation solution); rac-BMBA (10 mM), pyruvic acid (10 mM), PLP (0.5 g/L). Flow rate was maintained constant but contact time was varied by adapting the bed size.

It must be mentioned that the cofactor PLP had to be fed continuously during enzymatic reaction. Indeed, when no PLP is present in the reaction medium, almost no conversion occurred (4 % on A50_A-GA sample, although PLP was present during enzyme

immobilization step). This suggested that the cofactor did not stay into the active site and is then leached during flow transamination.

ATA-117 concentration in the impregnation solution was varied, keeping all other parameters of the enzyme immobilization constant (Figure 1 - 18). The yield increased with the concentration of the enzyme in the impregnation solution. However, the yield appeared to level off when the ATA-117 concentration in the impregnation solution reached 0.2 g/L.

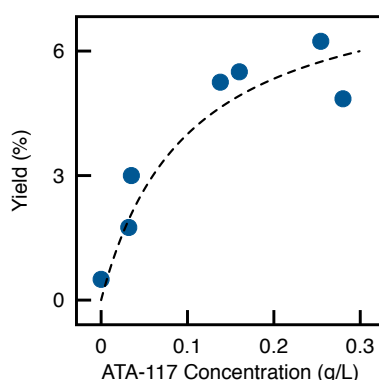


Figure 1 - 18 – Effect of ATA-117 concentration in impregnation solution on A50_A-GA biocatalysts efficiency. Experimental conditions: rac-BMBA (10 mM), pyruvic acid (10 mM), PLP (0.5 g/L). Curved line stands as a guide to the eye.

Before each flow reaction, reactors were washed with buffer to ensure that only the immobilized enzyme remain in the monolith when the reaction is started (Figure 1 - 19). First, the enzymatic solution (50 mL) was injected into the reactor (“in”) and the outflows were collected (“out”; 50 mL). Then, 50 mL of buffer is injected through the reactor and outflows were collected into 10 mL fraction volumes. The enzyme concentration was determined in each collected fractions by the adapted Bradford method (see Annexe 3). Only a small fraction of the injected enzymes may have been immobilized. After 20 mL of washing buffer, no enzyme was detected in the outflow, suggesting that the washing step was efficient.

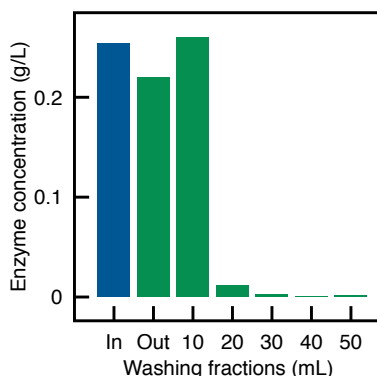


Figure 1 - 19 – Washing efficiency on A50_A-GA sample. Washing was satisfying as no enzyme was detected after 20 mL of washing buffer.

Looking at the rough estimation of the enzyme loading in this monolith (~1.2 mg), it is possible to estimate the residual activity of the immobilized enzyme, as compared to the free enzyme. The conversion obtained in flow is compared to the conversion of a homogeneous batch reactor containing the same amount of enzyme in the same conditions and after a period equal to the contact time. The ratio of the conversion in flow over the conversion in batch is the residual activity. The equivalent homogeneous batch should give a 37.5 % yield in the same conditions, so that the residual activity is estimated to 16 % in this case.

2.5 Applicability and robustness of the flow bioreactor

A specific experiment was carried out to demonstrate that the enzyme remains enantioselective when immobilized (Figure 1 - 20). In batch mode, ATA-117 showed an excellent enantioselectivity (see Figure 1 - 3). Thus, it is supposed that the immobilized enzymes will retain all its enantioselectivity. In the first part of the experiment, only S-BMBA was fed in the reactor (instead of the racemic BMBA). As expected, the BAP yield remains very low at the reactor flow outlet, corresponding exactly to the non-catalytic conversion occurring in the reagent solution in the sole presence of PLP. After 180 minutes, the racemic BMBA solution was injected in the reactor. After a 140-minutes lag time required time for the reaction medium to pass through the flow device, BAP yield reaches 6 % again. This demonstrates unambiguously that ATA-117 immobilized in the flow reactor remains enantioselective.

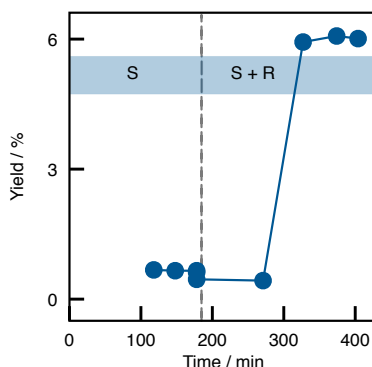


Figure 1 - 20 – TA-A50_A-GA enantioselectivity. Experimental conditions: ATA-117 (10 mg in impregnation solution), pyruvic acid (10 mM), PLP (0.5 g/L). With S-BMBA enantiomer, the yield is low and corresponds to the non-enzymatic reaction. When the racemic BMBA is injected in the reactor at 180 min, the yield raises up to 6 % after 2 hours, which is the time required for the solution to reach the outlet.

From an industrial perspective, it is important to ensure that the biocatalyst allows reaching total conversion, *i.e.* the actual kinetic resolution with only one BMBA enantiomer remaining at the outlet.

In that purpose, a catalyst with large bed size should be implemented in flow mode. We chose to use several small monoliths in series (to avoid handling one long monolith). The total bed size is chosen large enough to ensure total conversion, but not too long, to limit the pressure drop. By looking at the yield provided by one monolith (6% for TA-A50_A-GA, see Figure 1 - 15), it is chosen to implement 8 monoliths in series ($8 \times 6 \% = 48 \%$) that should provide almost total yield. Indeed, in our conditions, enzyme is saturated by its substrate and then, the conversion increases linearly with time (or contact time), up to a plateau (where the yield does not depend on time). Thus, eight functionalized monoliths were connected in series and then pre-activated with glutaraldehyde and loaded with the enzyme (TA-A50_A-GA samples, Figure 1 - 21). After a 200-minutes lag time required time for the reaction medium to pass fully through the flow device, the yield reached the maximum of 50 %, with excellent enantioselectivity, as attested by GC and chiral HPLC measurements (the *R*-BMBA enantiomer was not detected by chiral HPLC, thus the enantiomeric excess is assumed to be $\geq 99 \%$).

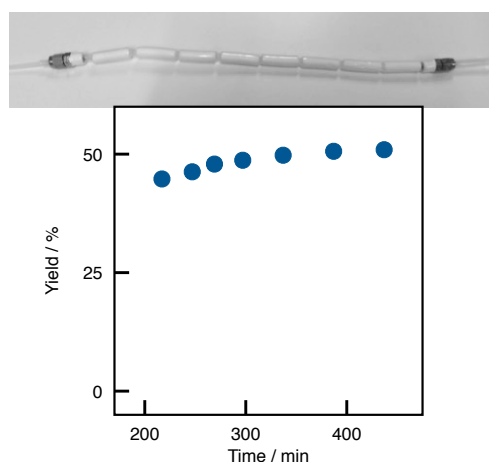


Figure 1 - 21 – Maximal yield using eight monoliths in series. (Up) Reactor made of eight TA-A50_A-GA samples in series in order to reach total conversion. (Down) Total conversion is reached by increasing contact time (eight TA-A50_A-GA samples in series). Experimental conditions: ATA-117 (10 mg in impregnation solution); rac-BMBA (10 mM), pyruvic acid (10 mM), PLP (0.5 g/L).

To assess storage stability and recyclability, a TA-A50_A-GA biocatalyst was tested in a classical flow reaction, which was interrupted after 4 h. The reactor was sealed at both ends (not purged) and stored at 4 °C for 16 h. Then, the reactor was again put in place in the flow set-up and fresh reaction medium was fed. The yield was practically as high as before cold storage (~ 5.5 % instead of ~ 6 %; Figure 1 - 22). After 5 h of reaction, the reactor was again removed, sealed and stored at 4 °C for 68 h. Then, it was tested again with fresh reaction medium and the final yield was still ~ 4.7 %. Thus, residual activity was 80 % after 4 days of cold storage. It indicates a good stability and recyclability of the biocatalysts. The low deactivation could be assigned to a slow enzyme leaching. However, no enzyme was detected in the outflows (when tested with Bradford reagent) and no activity was detected in the outflows. Then, this potential leaching is assumed to be very weak. Also, this slow deactivation could be assigned to a slow enzyme denaturation on the monolith surface.

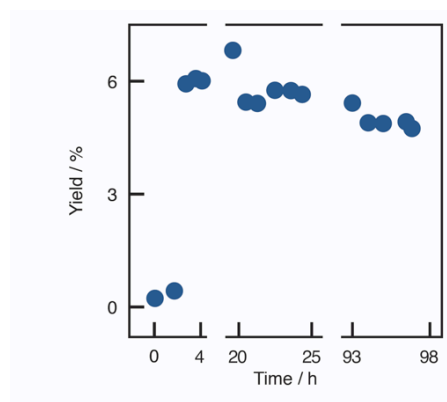


Figure 1 - 22 – Stability under cold storage. Experimental conditions: ATA-117 (10 mg in impregnation solution); rac-BMBA (10 mM), pyruvic acid (10 mM), PLP (0.5 g/L). Yield with TA-A50A-GA, flow reaction is carried out for periods of ~14 h interrupted by cold storage.

3 Conclusions

Macrocellular silica monolith have been prepared by a facile sol-gel route, and used as support for the immobilization of transaminases. Such robust materials are directly produced in the desired dimensions and shape, and they are easily incorporated in a flow set-up. The porous medium favoured the establishment of a plug flow regime and it is shown to withstand flow constraints. Enzyme immobilization was carried out successfully when using APTES-functionalized supports previously activated by glutaraldehyde. Once immobilized, transaminase enzymes retain their activity and their enantioselectivity. Moreover, the solid biocatalyst is shown to maintain its activity over prolonged reaction and storage times. Conversion yield can be tuned by adapting the contact time and no diffusional limitations were observed. This study shows that such heterogeneous biocatalysts exhibit all the required features for an enzymatic flow process.

However, as the residual activity was quite low, there was still room for improvement in particular regarding the functionalization process. Moreover, the transamination was performed in the kinetic resolution mode, thus not the way of industrial interest. Attempts to drive the equilibrium towards the asymmetric synthesis will be discussed in the Chapter V. Finally, one remaining problem is the need to continuously feed the PLP that represents a high cost for an industrial application. This issue is not addressed in this work, but must be resolved for an industrial application, e.g. using products

extraction devices, and by implementing a line for the recycling of aqueous waste.

Chapter II – Understanding and optimizing support functionalization and enzyme grafting

1 Introduction

In this chapter, we highlighted a non-uniform APTES grafting when using the Method A for functionalization (observed macroscopically and in IR and TGA). We have suspected that the bad APTES dispersion was due to the functionalization process. Thus, attempts to improve the APTES dispersion while maintaining satisfying enzymatic activity was conducted. Parameters that were assumed to govern the functionalization process were studied: the presence of water, the silanols availability at the silica surface, the impregnation method (wet or dry), and the APTES concentration.

We discovered a strong link between the working temperature during the enzyme immobilization and the activity of the biocatalyst. We highlighted that the temperature was a crucial parameter for maintaining the functional homo-dimeric structure.

Improvements towards the enzyme immobilization process were also considered, in particular, the reinforcement of the covalent bond (through imines reduction).

2 Results and discussion

2.1 Functionalization (Method A) and APTES dispersion

In the first chapter, we demonstrated the feasibility of using immobilized ATA-117 transaminases on macro-cellular silica monoliths for the enantioselective transamination in continuous flow. In the best conditions, using a monolith functionalized with APTES according to the « Method A », we obtained a stable yield of 6 %, with excellent enantioselectivity and stability. However, after the reaction, biocatalysts were cut lengthwise, and a brownish crust was observed (Figure 2 - 1).

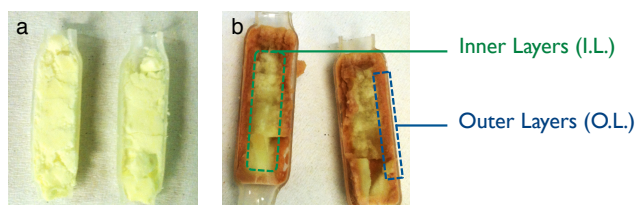


Figure 2 - 1 – APTES dispersion of samples functionalized by the Method A, observed after the flow enzymatic reaction. (a) TA-A0_A-GA, (b) TA-A10_A-GA. Outer layers (O.L.) were collected by scratching the monolith over 1 mm thickness. Inner layers (I.L.) were the cores of the scratched monoliths.

We have suspected that this uneven colour distribution was linked to the method of functionalization, which was not optimized yet. This phenomenon must be linked to the reaction between free amino groups in the grafted aminopropyl groups and the reactant BMBA in the presence of glutaraldehyde (Figure 2 - 2). This visually suggests that the APTES dispersion throughout the monolith is not homogeneous.

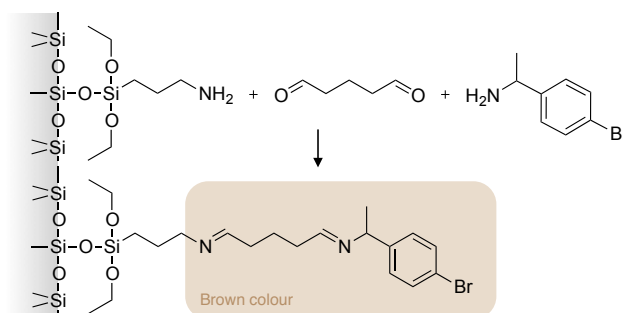


Figure 2 - 2 – Origin of the brownish colour. Brown colour produced by the reaction between the amino moiety and BMBA in the presence of glutaraldehyde.

ATR-FTIR and TGA analyses were performed on the inner and outer layers of a monolith functionalized by Method A. Outer layers were collected by scratching the monolith over 1 mm thickness. Inner layers were the cores of the scratched monoliths (see Figure 2 - 1).

TGA analyses (Figure 2 - 3, left) confirmed an uneven APTES dispersion in the monolith for the Method A, since the weight loss (assigned to grafted aminopropyl moieties) were much higher from the sample scratched from the outer layers. This was also confirmed by ATR-FTIR analyses (Figure 2 - 3, right) as C-H stretching band absorbance was detected around 2900 cm^{-1} only for the outer layer of the Method A-functionalized monoliths and not even visible on the sample coming from the inner layer.

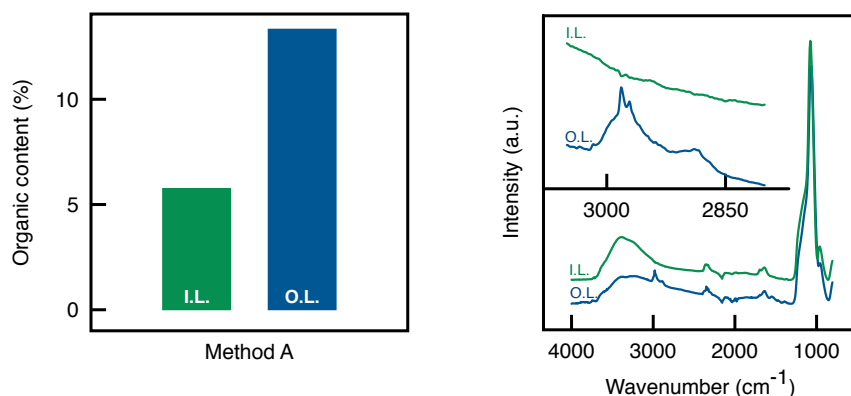


Figure 2 - 3 – TGA and ATR-FTIR spectra of Method A-grafted samples. (Left) TGA analyses of A50_A showed a heterogeneous APTES dispersion in the monolith for the Method A (more organic functions assigned to APTES moieties in the outer layer). (Right) C-H stretching band absorbance was detected around 2900 cm^{-1} only for the outer layer of the sample A50_A.

Our interpretation of the heterogeneity of the APTES grafting is linked to the method of functionalization. During the functionalization process with the Method A, the monolith was soaked into a large excess of the grafting solution. Thus, even if the whole porosity of the monolith is filled with an APTES solution, the external environment is also composed of the same APTES solution. During the 24h of functionalization, new APTES molecules could continuously diffuse from the excess solution and enter into the monolith, thereby functionalizing mostly the outer part of the monolith. In this way, an APTES gradient is established.

Also, it appears that quantifying APTES in the monolith is a touchy task. Indeed, first, we do not know if APTES was reacted with

one, two or three silanols. If one APTES reacts only with one silanols (as in Figure 2 - 2), that leads to potentially three times more APTES in the monolith than if APTES molecules react with three silanols. Also, in the presence of water, the amino function can be protonated. The protonated amine could be folded towards the silica surface (electrostatic interactions with silanols) or ethoxy group (from another APTES molecule) [182]. Thus, the amino function is not available anymore for enzyme immobilization. In any cases, the presence of water (and its pH) influences this phenomenon. Moreover, at this stage, the amount of APTES that effectively penetrates all the (micropores is not know (however, the enzyme will not be able to enter into the micropores). Finally, the amount of APTES that is washed away by the washing steps is not known either.

It appears reasonable to put forward that a more homogeneous APTES functionalization throughout the monolith would lead to a more effective enzyme immobilization and therefore, to higher biocatalytic performance. This is why Methods B, C, and D are proposed as attempts to improve the quality of the APTES functionalization step.

2.2 Improving APTES-functionalization

2.2.1 Functionalization in « dry » conditions (Method B)

Method B (see Experimental, Section 2.1.2.3) was implemented in an attempt to obtain better APTES dispersion into the monolith. The monoliths were briefly dipped in the grafting solution under vacuum and removed from the solution right after the effervescence step. The solution was more concentrated (500 mM) in APTES than in Method A. The monoliths were then disposed on a grid and maintained in a toluene-saturated atmosphere for 24 h (in a closed-desiccator containing a toluene flask). They followed the same procedure for the washing, drying, storage, enzyme immobilization, and they were tested in the kinetic resolution of BMBA (*vide infra*). The monoliths were cut lengthwise after the catalytic reaction in flow. APTES dispersion appeared to be homogeneous throughout the monoliths as no crust was observed (Figure 2 - 4). Instead, a homogeneous yellow colouring was observed throughout the monolith. In order to confirm the good APTES dispersion, ATR-FTIR and TGA analyses were performed on the inner and outer layers of the samples functionalized by Method B. TGA analyses (Figure 2 - 5, left) showed that the Method B achieved a homogeneous functionalization throughout the monolith since the same organic content was detected in both layers. This was confirmed by ATR-

FTIR analyses (Figure 2 - 5, right) as the same signal (C-H stretching band absorbance around 2900 cm^{-1}) was detected in both layers with similar intensity, for the samples functionalized by Method B. Indeed, as all the pores were filled by APTES solution, and as there was no APTES excess during the functionalization process, the APTES dispersion throughout the monolith was supposed to be homogeneous.

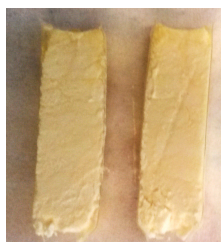


Figure 2 - 4 – Sample functionalized by the Method B, cut lengthwise. Functionalization Method B led to a good APTES dispersion into monoliths (TA-A100_B-GA sample).

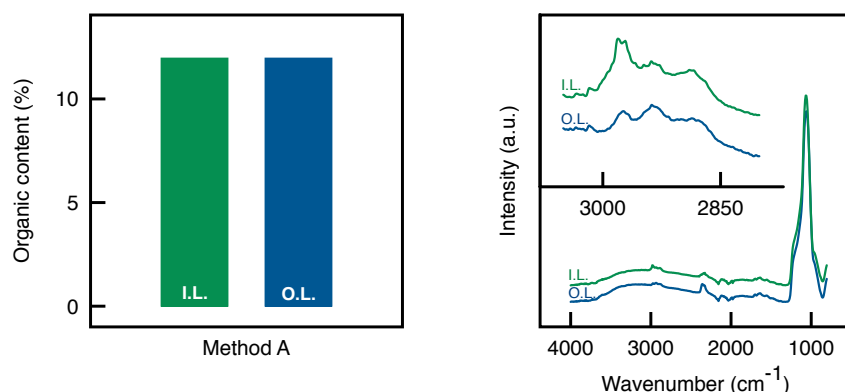


Figure 2 - 5 – TGA and ATR-FTIR spectra of Method B-grafted samples. (Left) TGA Analysis of A500_B sample: Method B allowed a good APTES dispersion (same organic content in both layers). (Right) ATR-FTIR: C-H stretching band absorbance was detected around 2900 cm^{-1} in both layers with similar intensity for Method B (A500_B).

TA-A500_B-GA biocatalysts were prepared independently following strictly the same procedure and were tested in the kinetic resolution of BMBA. The catalysts showed on average a yield of 11 % (much higher than the 6 % yield obtained with Method A samples, Figure 2 - 6). A 2 mg loading was obtained in average for A500_B-GA samples (RSD was 57 % for the loading measurement). This loading should give a 41.5 % yield in a homogeneous batch. As yields ranged from 5

to 15 %, residual activity estimation ranged from 12 to 36 % (residual activity for TA-A50_A-GA sample was 16 %, see Chapter I, Section 2.4).

Thus, the better dispersion of APTES throughout the monoliths seems to be beneficial for the enzyme anchoring. However, the repeatability was poor (RSD was 40 %). This may be assigned to a relatively high variability of silanols availability on Si(HIPE) monoliths from batch-to-batch. Indeed, according to Zhuravlev [136], dehydroxylation of the silica surface should be expected to occur during monolith calcination. In contrast, water from air humidity could slowly rehydroxylate the silica surface before the functionalization step. This rehydroxylation step was not controlled at all in Method B and may have varied from one experiment to the other.

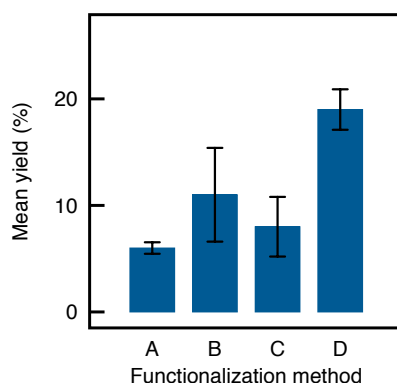


Figure 2 - 6 – Mean yields and RSD for flow reactions depending of functionalization method used. Experimental conditions: ATA-117 (10 mg in impregnation solution), rac-BMBA 10 mM, sodium pyruvate (10 mM), PLP (0.5 g/L). Samples are TA-A50_A-GA, TA-A500_B-GA, TA-A500_C-GA, TA-A500_D-GA.

2.2.2 Rehydroxylation prior to silanization (Method C)

During Si(HIPE) synthesis, materials are calcined at high temperature (650 °C) to ensure a good mechanical resistance of this highly porous silica structure [183]. After the synthesis, Si(HIPE) are exposed to ambient humid air. Then the water contained in the air could induce a slow rehydroxylation at the silica surface. As the humidity is not controlled, we suspected that the exposure to ambient air could lead to non-controlled silanols availability at the surface. However, those silanols are the anchoring points for the grafting of the aminopropyl groups. Thus, Method C (see Experimental, Section 2.1.2.3) was used in order to get more silanols at the surface and to

control this rehydroxylation step. Then, the functionalization by APTES is identical as in Method B.

Si(HIPE) monoliths re-hydroxylation was inspired by Zhuravlev [136] (Figure 2 - 7): after soaking in water at ambient temperature and under vacuum (to force the water entering into the pores), monoliths were heated at 100 °C in a closed glass recipient. In order to remove excess water, while maintaining a large amount of hydroxyl groups, monoliths were then dried at 190 °C under vacuum.

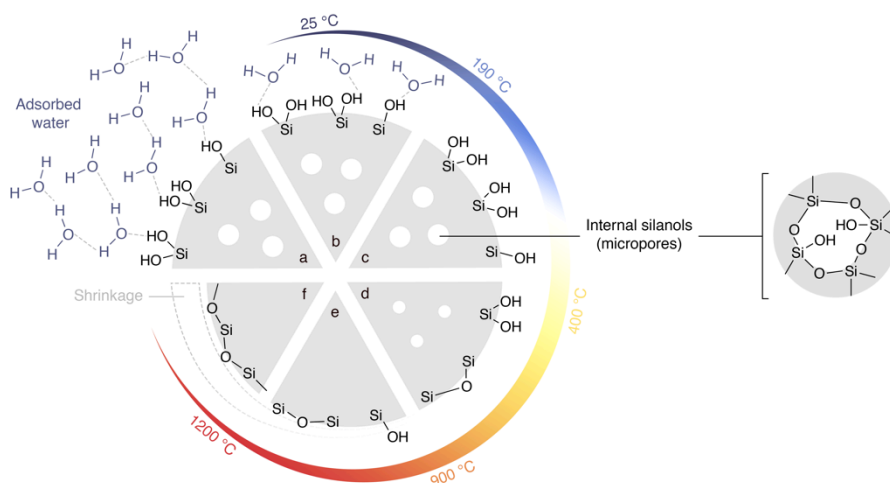


Figure 2 - 7 – Silica heated under vacuum: Zhuravlev model. (a) Hydrated silica at 25 °C. Many silanols are present at the surface as well as internal silanols (in micropores). Water is in excess and forms multilayers. When heated under vacuum (b), silica gradually loses surface water. (c) At the key temperature of 190 °C, silica is totally dehydrated but still contains many silanols (the maximum). (d) Beyond 190 °C, silanols condensation into siloxane bridges gradually occurs at the surface. Over 400 °C, this condensation is irreversible. (e) Over 900 °C, condensation of internal silanols and of most of the surface silanols occurs. (f) Finally, by heating in vacuum up to 1200 °C, silica surface loses its last silanols into siloxanes. Shrinkage is observed beyond 400 °C.

Temperature-programmed desorption of water has been performed on rehydroxylated samples. Samples are gradually heated under argon atmosphere. Above 400 °C, water desorption is assigned to silanols condensation. Figure 2 - 8 (left) shows higher water desorption attributed to silanols condensation for the rehydroxylated sample, suggesting that the rehydroxylation procedure indeed allowed creating more silanols at the silica surface.

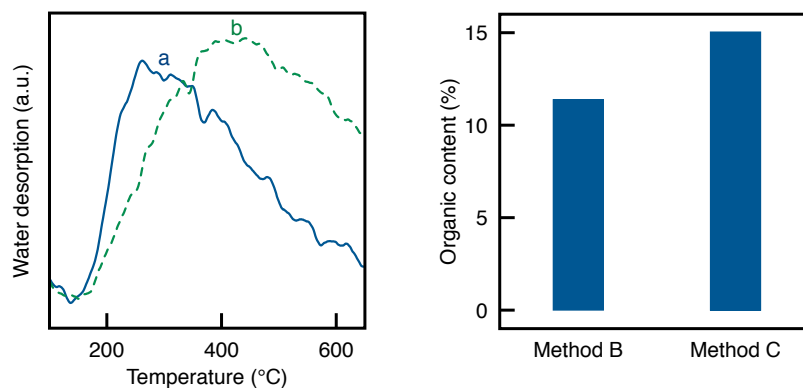


Figure 2 - 8 – Effect of rehydroxylation on silanols availability and APTES grafting efficiency. (Left) Thermo programmed water desorption of (a) Si(HIPE) and (b) rehydroxylated Si(HIPE). (Right) TGA analyses of A500_B and A500_C samples. More organic functions were detected on the rehydroxylated samples. This suggested that the rehydroxylation process allowed featuring more silanols at the surface.

Benefiting from a higher surface density of hydroxyl groups, it was expected that more APTES molecules could be grafted. On the one hand, TGA analyses confirmed the presence of a larger organic content on the samples that have been rehydroxylated (A500_B *versus* A500_C, Figure 2 - 8, right). On the other hand, ATR-FTIR analyses did not allow evidencing a higher intensity for organic moieties in the sample prepared by Method C as compared to Method B (not shown).

Efficiency of TA-A500_C-GA biocatalysts was assessed in flow mode (Figure 2 - 6). They showed a slightly lower yield than the A500_B-GA samples (8.4 % in average) and still a low repeatability (RSD was 35 %).

Thus, although the Method C seems to allow increasing the silanol availability on the silica surface prior to the APTES grafting, it has not allowed to increase drastically the efficiency of the APTES functionalization and the biocatalytic performance obtained after subsequent enzyme grafting were not improved either. Moreover, the repeatability of the catalytic performance remained poor. Thus, the controlled rehydroxylation seems not to be a critical parameter for the functionalization process, unlike what was expected.

2.2.3 Controlling water availability (Method D)

The presence of water during silanization reactions is known to be a critical parameter. Indeed, a little amount of water is needed to catalyse the silanization reaction [139]. But, the presence of higher amounts of water is known to provoke the polymerization of APTES molecules leading to a non uniform APTES distribution that could explain the variability in the biocatalytic reaction [137,184]. Regarding the functionalization of the silica monoliths, it is suspected that these highly porous solids adsorb water (from ambient humid air) after the calcination step, and as this is not controlled in any way, it is identified as a possible cause for a lack of reproducibility in the functionalization step. Also, dried toluene used for APTES grafting could absorb water from ambient humidity, as soon as the dried toluene bottle is opened. This phenomenon is not controlled either. Thus, water availability during the functionalization process is difficult to control from both sides: adsorbed water in the monolith and dissolved water in toluene.

Therefore, Method D was applied as an attempt to strictly control the presence of water throughout the functionalization process. Si(HIPE) monoliths were dried at 120 °C under vacuum for 24 h in order to remove excess water from the monolith. After cooling under vacuum, monoliths were directly functionalized using water-saturated toluene as the solvent, without intermediate storage. Therefore, water inputs are totally controlled both in the monolith and in the APTES solution. The preparation was repeated independently on 4 different silica monoliths following strictly the same procedure. We must mention that the water concentration, although controlled, was not optimized in the monoliths. The use of a room with controlled humidity level could allow identifying the optimal water content for the functionalization process.

ATR-FTIR analyses (Figure 2 - 9, left) showed a slight increase in C-H vibration band absorbance with the presence of APTES. Moreover, primary amine scissoring band appeared (at 1570 cm⁻¹) [175] with APTES concentration. TGA analyses of samples functionalized by the Method D confirmed that the organic content was higher than in the blank sample (which has undergone the same functionalization procedure but without APTES; Figure 2 - 9, right). However, the organic content did not increase markedly with the APTES concentration and instead seemed to level off, suggesting that monoliths are saturated by APTES moieties, already upon impregnation with 500 mM APTES solution.

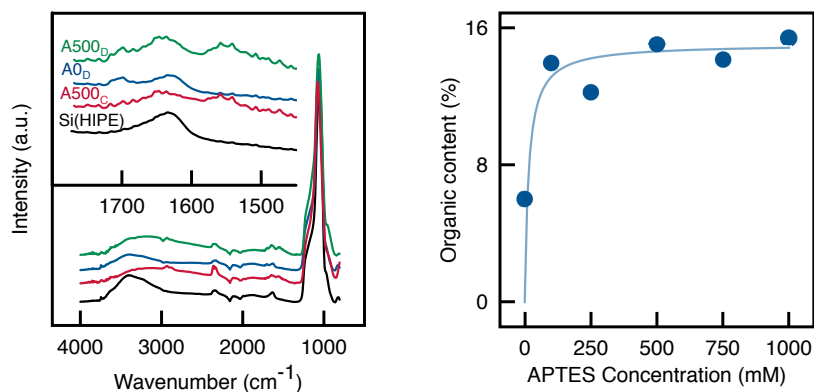


Figure 2 - 9 – ATR-FTIR spectra and TGA of Method D-grafted samples. (Left) ATR-FTIR spectra of Si(HIPE), A0_D and A500_D. The occurrence of NH₂ scissoring band absorbance (1570 cm⁻¹) suggests the presence of aminopropyl groups grafted to monolith surface for A500_D sample. (Right) TGA analyses of Method D functionalized samples. An increase in organic content is observed up to 250 mM APTES, then followed by a saturation plateau.

When comparing the Methods C and D, it appears that functionalized monoliths exhibit the same organic content (both A500_C and A500_D have 15 % organic content when analysed in TGA; Figure 2 - 8, right, and Figure 2 - 9, right). The IR spectra are also similar for both methods (Figure 2 - 9, left): primary amine scissoring bands appear at 1570 cm⁻¹. It seems then that the Method D did not bring more APTES at the monolith surface. A rough quantification of APTES grafted on A250_D monolith (when the monolith seems to be saturated in APTES) is attempted: assuming that all the porous volume (see Experimental, Section 2.3.2.2) is filled by APTES solution, and assuming that no APTES is washed away during the washing steps (see Experimental, Section 2.1.2.3), the maximal APTES amount effectively grafted per monolith (150 mg) is $5 \cdot 10^{-4}$ mol.

When the enzyme was immobilized on these monoliths, the loading remained relatively low (Figure 2 - 10, left). In fact, it was lower with all the APTES-functionalized monoliths than with the blank sample (TA-A0_D-GA) which has undergone the same procedure but in the absence of APTES (and for which simple adsorption is the sole immobilization mode). The highest enzyme loading on APTES-functionalized monoliths (~0.7 mg) was obtained with the functionalization solution of 500 mM of APTES.

Despite relatively low enzyme loading, these biocatalysts prepared from monoliths functionalized via Method D showed

relatively high performance (Figure 2 - 10, right). The yield reached 19 % in average with a good repeatability (e.g. RSD was 9.2 % for TA-A500_D-GA sample). Yields increased quite linearly with the APTES concentration in the functionalization solution, up to 250 mM. However, at higher APTES concentration, the catalytic yield dropped.

The relation between enzyme loading and the activity of the monolith is plotted in Figure 2 - 11. For APTES-functionalized samples, the yield increases when the loading increases up to a plateau around 20 % yield. This plateau could suggest that the monolith surface is both saturated in APTES and in transaminase. However, multi-layer of enzyme could still be immobilized on the monolith (and thus, the loading increases), but the yield stays stable as new multilayers hide transaminases that were already immobilized on the surface. The only value that lands out of the trend corresponds to the blank sample (TA-A0_D-GA) for which the loading is remarkably high (~1 mg), but the yield is very low (3.4 %). On the one hand, it seems that the enzyme can be immobilized in large amounts by simple adsorption. On the other hand, as the yield is low, it is suggested that enzymes are deactivated when simply adsorbed. Indeed, it is well known that enzyme conformation, crucial for catalytic properties, could be altered during simple adsorption [98,107]. In contrast, when APTES is grafted on the silica surface, even in low concentration, the loading is clearly lower, suggesting that enzymes can only be immobilized on amino sites activated by glutaraldehyde (and not elsewhere). Yet, the yield is significantly higher than with the blank, suggesting that APTES could confer a favourable environment for the immobilized enzyme (preventing the deformation of the structure for example). Hydrophobic effects brought by APTES grafting have been already highlighted in the literature [185], and could possibly explain this result. APTES functionalization would limit the amount of enzymes that can be immobilized, but would at the same time favour enzyme activity, for example by stabilizing its conformation. The impact of surface hydrophobicity on transaminase immobilization and performance is extensively studied in the Chapter III.

When comparing the yields obtained with monoliths grafted by the Methods C and D, we observed that the Method D allows a greater yield as A500_C and A500_D exhibit respectively 8.4 % and 19 % mean yield. Thus, despite similar analyses in IR and TGA, Method D-functionalized samples offer a better biocatalytic efficiency. That may be due to a better control on the APTES dispersion throughout the monolith with the last functionalization method.

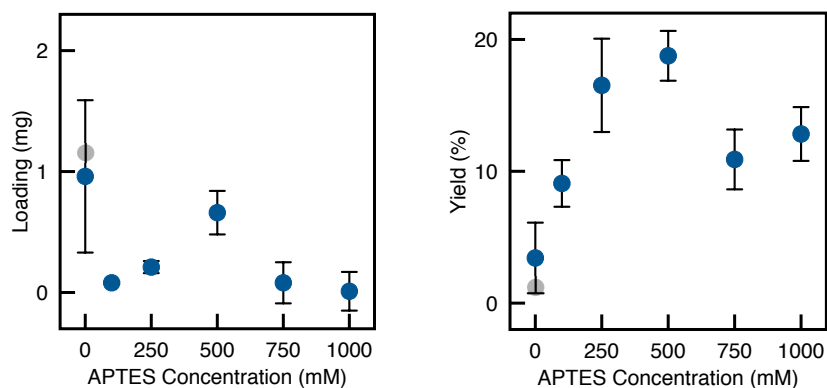


Figure 2 - 10 – Loading and Yield of Method D-grafted samples. (Left) Loadings, for the same samples, reached a maximum at 0.96 mg, and then dropped. (Right) Yields for TA-A0_D-GA to TA-A1000_D-GA samples. The yield increased linearly with APTES concentration up to 250 mM. Shaded dots (●) represent the yield and loading for TA-Si(HIPE) sample. Experimental conditions: ATA-117 (10 mg in impregnation solution), rac-BMBA 10 mM, sodium pyruvate (10 mM), PLP (0.5 g/L).

Regarding to the Figure 2 - 10 (left), it seems that the maximal loading capacity of our catalyst would reach 1 mg. If supposing that the ATA-117 stock is pure (we know it is not the case, see Chapitre I, Section 2.1), $2.7 \cdot 10^{-8}$ mol of ATA-117 are immobilized on each monolith. This is much below the maximal APTES concentration ($5 \cdot 10^{-4}$ mol/A250_D monolith). So, either the APTES concentration in the monolith was overestimated (that's probably the case as the calculation does not take account from the APTES leached during washing steps), or the enzyme loading is underestimated (but due to issues on loading measurement, it is hard to decide).

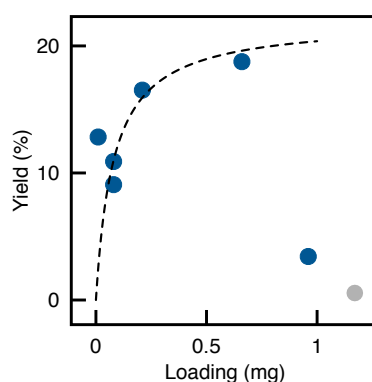


Figure 2 - 11 – Relation between yield and loading for samples functionalized with the Method D. Dotted curve is only a guide to the eye. Shaded dot (●) represents the yield and loading for TA-Si(HIPE) sample.

In conclusion, Method D seemed to allow a better control on the functionalization step, by strictly controlling the amount of water that is involved in the process. This confirms the crucial role of water in such silanization reactions. With this method in hand, it is possible to obtain monoliths, which exhibit higher catalytic yield, together with a highly improved repeatability. An APTES concentration of 250-500 mM was identified as the best compromise to ensure that enough organic moieties are grafted and to optimize both the enzyme loading and the catalytic yield in the flow reaction. Table 3 summarizes the main improvements performed concerning the functionalization method.

Table 3 – Functionalization improvements – From Method A to D.

Sample	Dispersion	Mean yield (RSD)	Mean organic content *	Estimated enzyme loading (Bradford method)
A50 _A	Poor	6 % (9%)	11 %	1.2 mg
A500 _B	Good	11 % (40 %)	7 %	2 mg
A500 _C	Good	8 % (35 %)	12 %	1.7 mg
A500 _D	Good	19 % (9.2 %)	15 %	0.7 mg

* TGA of entire monolith

2.3 Improving the immobilization efficiency

2.3.1 Chitosan as an alternative to the APTES grafting

A greener alternative to the functionalization process was considered: Coating the silica surface with chitosan (biopolymer derivative of chitin). Chitosan features amines functions [186] that can be used (i) to immobilize the polymer on the silica surface [144]; (ii) to immobilize enzymes on the chitosan surface [110–112] (either by adsorption assisted by electrostatic interactions, or by covalent grafting with glutaraldehyde, Figure 2 - 12). Indeed, in acidic medium (below pH 6.5), amine groups are positively charged, and then chitosan is water-soluble. In neutral or alkaline conditions, chitosan is insoluble [187].

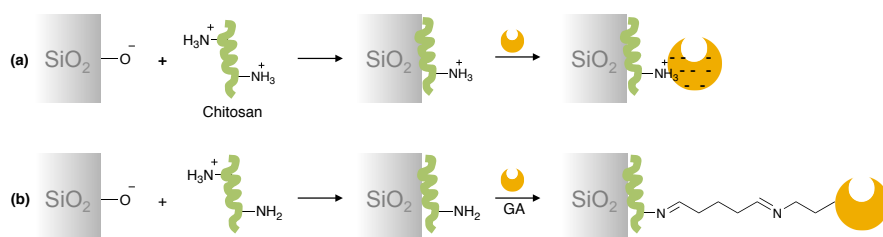


Figure 2 - 12 – Chitsosan as an alternative to APTES grafting. Chitosan adsorption on silica surface (by taking benefit of negatively charged silanols) and enzyme immobilization: (a) Enzyme adsorption assisted by electrostatic interactions, (b) covalent grafting with glutaraldehyde (GA).

This part of the project was developed by Ir. Thibaut Huybrechts during his master thesis. First, interactions between ATA-117 and chitosan particles were studied in batch conditions. Unfortunately, enzyme immobilization on those particles was inefficient due to a low specific surface area on chitosan particles. Then, we tried to spread the chitosan over the Si(HIPE) silica surface to overcome this situation. After enzyme immobilization, a strong deactivation was detected in flow mode. Finally, conversion was very low. In spite of reduced enzyme leaching through NaBH_3CN treatment (in order to reduce hydrolysable imine bonds formed during glutaraldehyde pre-activation), the system could not be improved. Moreover, troubles to push the liquid through the monolith were encountered, and assigned to a chitosan swelling [188,189].

2.3.2 Effect of temperature during enzyme immobilization

Classically, enzymes are manipulated in cold conditions to prevent natural deactivation (e.g. due to protease activity or bacterial growth) [87,190]. Accordingly, enzyme immobilization was systematically carried out at 4 °C, using ice packs to cool the enzyme solution and the monolith. Enzyme immobilization was also tested at 30 °C instead of 4 °C on the A250_D support. In the following, the suffix “30d” is added to the name of the biocatalysts prepared by immobilization at 30 °C. A higher enzyme loading was measured for TA-A250_D-GA-30d (0.7 mg) as compared to the TA-A250_D-GA (0.2 mg). Moreover, the resulting biocatalytic yield was significantly higher with TA-A250_D-GA-30d (Figure 2 - 13).

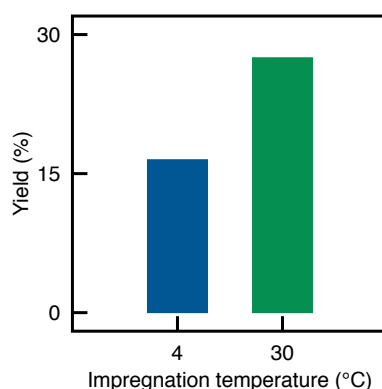


Figure 2 - 13 – Effect of impregnation temperature on catalytic efficiency. Samples are TA-A250_D-GA and TA-A250_D-GA-30d. Experimental conditions: ATA-117 (10 mg in impregnation solution), rac-BMBA (10 mM), sodium pyruvate (10 mM), PLP (0.5 g/L).

Transaminases are homo-dimeric enzymes, with the active site formed only when two monomers assemble (Figure 2 - 14) [54]. When the environment gets cold, some multimeric enzymes may reversibly dissociate, leading to a so-called “cold dissociation”, as reviewed by Privalov [191]. According to Privalov hypotheses, the non-polar residues located on the enzyme surface are hydrated by water molecules. This hydration is highly favourable. Unlike all the expectations, “the Gibbs energy of hydration is negative and increases in magnitude at a temperature decrease” [191]. Thus, the more the enzyme solution gets cold, the more this non-polar area is hydrated, and then the protein unfold to expose more non-polar residues to water. In the case of transaminases, a highly non-polar area is located at the interface of monomers. If the dimer dissociates, the transaminase loses its activity (as dimerization is needed for activity).

This result can be rationalized in the light of the “cold-dissociation” phenomenon. When immobilization was practiced under cold conditions, mainly inactive monomers could have been immobilized on the monolith. Once two monomers are immobilized on different grafting sites, reforming the active dimer is impossible. Moreover, the immobilization of monomer could have occurred through the lysine residue located on the active site, thereby locking the enzyme with the active site towards the support surface, blocking the access for the reactants. On the contrary, during « 30d » immobilization, mainly dimers would be present in the solution, and immobilized as such, in their active form. Also, as the dimeric form

protects the active site (formed between the two associated monomers), immobilization via the active lysine is avoided.

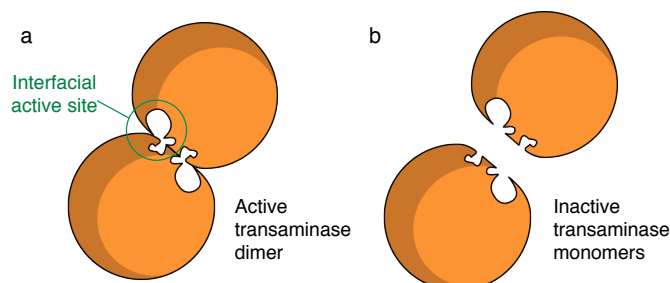


Figure 2 - 14 – Schematic representation of monomer dissociation that would occur upon cold conditions. Transaminases are dimeric enzymes. (a) Active sites are located on the interfacial area. (b) When the environment gets colder, monomers are suspected to undergo a reversible dissociation, leading to a “cold dissociation”.

Another explanation of this “deactivation” at low temperature is a heat-dependant rate of enzyme immobilization by covalent grafting: at 30 °C the binding between amines (from APTES and from enzymes lysine residues) and glutaraldehyde is faster, thus, more enzyme could be immobilized on the surface. At this stage, it was not possible to decipher between the two hypotheses to explain the lower performance obtained after enzyme loading in cold conditions.

It must be mentioned that optimization on impregnation temperature was not conducted. Such optimization could allow identifying an ideal impregnation temperature. We expect that over 30 °C (the optimal temperature for transamination reaction), the enzyme could undergo denaturation.

2.3.3 Imines reduction

As mentioned in the Chapter I, Section 2.5, the flow yield is relatively stable over a long period of time. Yet, a slight deactivation is observed over time: TA-A250_D-GA-30d samples exhibit 85 % of its initial activity after 300 minutes on stream (Figure 2 - 15, left). This can be an indication of a progressive deactivation of the immobilized enzymes over time. Also, even if no enzyme activity was detected in the outflows, this decrease in activity could possibly be assigned to a slow leaching of the immobilized enzymes from the monolith surface. Indeed the imine bond (Figure 2 - 15, right) can in principle be hydrolysed during reaction in water. A classical strategy to avoid such reaction is to reduce the imine bond to the amine, using for example

sodium cyanoborohydride as a reducing agent [93,115,192–194]. NaBH_3CN is classically used for the reduction of imine bond, when working with enzymes [115].

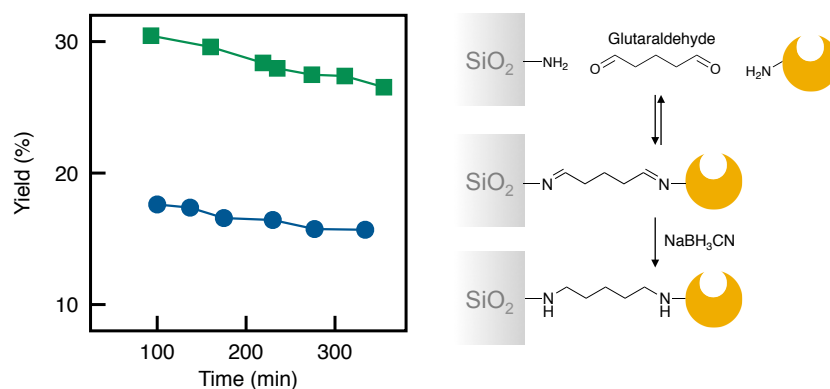


Figure 2 - 15 – Reduction of imine bonds for stability enhancement. (Left) Deactivation over time for TA-A250_D-GA-30d: (■) reference (no reduction); (●) reduction (Red3). (Right) Transaminases are immobilized through a reversible covalent bond (amines activated with glutaraldehyde, forming imines). Reducing imines into secondary amines leads to irreversible bonds. Experimental conditions: ATA-117 (10 mg in impregnation solution), rac-BMBA (10 mM), sodium pyruvate (10 mM), PLP (0.5 g/L), NaBH_3CN (0 or 50 mM).

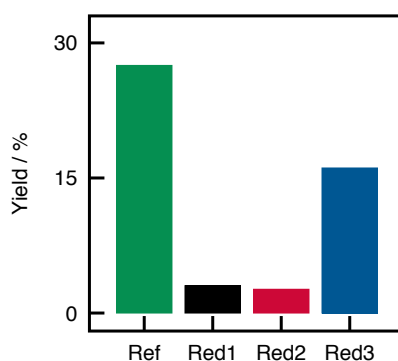


Figure 2 - 16 – Effect of imines reduction on A250_D-30d samples yields. “Ref” stands for reference sample (no reduction). Experimental conditions: ATA-117 (10 mg in impregnation solution), rac-BMBA (10 mM), sodium pyruvate (10 mM), PLP (0.5 g/L), 30 °C. Ref (no NaBH_3CN); Red1 (NaBH_3CN 50 mM (15 mL, 1.4 mL/min) injected after enzyme immobilization step); Red2 (NaBH_3CN 50 mM (15 mL, 1.4 mL/min) after enzyme immobilization step and PLP washing step (50 mL phosphate buffer, pH 8, 1.4 mL/min)); Red3 (NaBH_3CN 50 mM injected in the reaction medium, 0.11 mL/min).

In a first attempt (Red1), the reducing agent was injected right after the enzyme immobilization (after washing). In this case, a poor activity level was obtained as compared to the reference experiment without reduction (Figure 2 - 16, Red1). We suspected that this deactivation was caused by an enzyme denaturation in the presence of NaBH_3CN . In a batch process, we indeed observed that NaBH_3CN strongly deactivated the transaminases (see Annexe 4).

Indeed, after extended investigations in the literature, it seems that PLP-dependent enzymes deactivation by cyanoborohydride has already been observed [195,196].

The first step of the transamination reaction mechanism (see Introduction, Section 2.2.2) is the formation of an imine between the aldehyde group of the cofactor (PLP) and the essential primary amine of the enzyme active site (from a lysine residue). The results suggest that the reducing agent could attack the imine bond, leading to the irreversible formation of a secondary amine, permanently locking the cofactor into the active site. Then the transaminase is deactivated.

A second attempt was conducted to reduce imine bond on immobilized transaminases. First, transaminase solution (containing PLP in the buffer) was injected through the reactor. Then, the PLP was washed prior to the reduction in order to avoid locking it into the active site. Finally, the reducing agent was washed prior to the injection of reactants (containing PLP). However, this procedure also led to poor yield (Figure 2 - 16, Red2).

By looking back at the batch experiments (see Annexe 1), we observe that the reaction medium could act as a “protective agent” for the active site: no deactivation occurred when the reducing agent was added during the transamination. One possible explanation is that the reactants (having a good affinity for the active site and being present in relatively high concentrations, well above the K_M) tend to protect the active site simply by keeping it busy, and less accessible for the reducing agent. Thus, in a last attempt to reduce imine bond while keeping biocatalysts active (Red3), the reducing agent was fed to the flow reactor during the flow transamination. A satisfying activity was observed (Figure 2 - 16). Yet, the yield was still much lower than for the reference sample (no reduction), showing that the negative impact of the reducing agent on the active sites is still present in this case. In relative terms, however, the reduction (Red3) would allow to very slightly decrease the deactivation on stream (Figure 2 - 15, left) possibly by decreasing the enzyme leaching.

In conclusion, transaminase immobilization step is a milestone for biocatalytic efficiency. Indeed, a cold sensitivity has been highlighted (monomer dissociation). Enzyme immobilization at 30 °C allowed to retain more activity during flow transamination. Reducing imine bonds between enzyme and surface has been considered in order to prevent from enzyme leaching (through imines hydrolysis). However, the imine reduction caused an irreversible biocatalysts active sites deactivation. The Table 4 summarizes the main improvements performed concerning the immobilization step.

Table 4 – Immobilization improvements

Sample	Mean yield (RSD)	Loading (Bradford method)
A50 _A	6 % (9 %)	1.2 mg
A250 _D	16.5 % (21.5 %)	0.2 mg
A250 _D -30d	27.5 % (15.9 %)	0.7 mg

3 Conclusion

In conclusion, we identified essential parameters for good catalytic performance: the functionalization mode (dry *versus* wet), the water concentration during the functionalization process and the immobilization temperature. In practice, briefly dipping the monolith in the functionalization solution then aging in a solvent-saturated atmosphere allowed to obtain a good APTES dispersion throughout the entire monolith (confirmed by IR and TGA). Total water control during the process (by using dried monoliths and water-saturated solvent) is mandatory to minimize batch-to-batch variability (that affects enzyme immobilization and biocatalysts efficiency).

We highlighted a strong beneficial effect of carrying out the immobilization at 30°C instead of 4°C. An attempt to reduce the covalent bond was performed in order to minimize the deactivation over time, attributed to slight enzyme leaching. It appeared that transaminases are irreversibly deactivated by reducing agent (that affect their active site).

Chapter III – Design of hydrophobic enzyme-carriers and impact on catalytic properties

1 Introduction

The control on the hydrophobic/hydrophilic balance of catalyst surface is an important research topic in the broad field of heterogeneous catalysis. Indeed, tuning the hydrophilic or hydrophobic character of a support can help in the preparation of heterogeneous catalysts, especially for the optimized immobilization of active species or precursors [197,198]. Also, the surface polarity can play a role in the adsorption and desorption phenomena for both reactants and products and then, locally shifts the thermodynamic equilibrium of the catalysed reactions, thereby affecting the catalyst selectivity. In some cases, tuning the surface polarity – for example by adding hydrophobic groups on a hydrophilic surface – confers a larger stability in reaction conditions [114].

In the case of heterogeneous biocatalysis, the polarity of the enzyme carrier is of utmost importance. Most of enzymes exhibit hydrophobic patches on their surface [113]. Therefore, the immobilization of such enzymes could in principle be enhanced when immobilized on hydrophobic carrier. Indeed, hydrophobic interactions could reinforce the immobilization, possibly in combination with other kind of links, *e.g.* electrostatic or covalent interactions. Moreover, the presence of hydrophobic functions around the enzyme environment could help the catalytic act. Lipases immobilized on hydrophobic resins exhibiting a higher activity than in batch mode is a famous example when hydrophobic interactions between the enzyme and the carrier enhance the activity of immobilized enzyme [199].

ATA-117 transaminase exhibits 59 % hydrophobic residues on its surface, as calculated based on its published structure [51], using PISA tool [60]. So it is assumed that bringing hydrophobicity on the carrier could influence the immobilization yield and/or the catalytic efficiency. On the contrary, hydrophobic surfaces could alter the protein by a potential monomer dissociation (the interface between monomers is highly hydrophobic), or could confer a deleterious orientation of the enzyme on the surface (*e.g.* blockage of the active site stuck against the carrier wall). Thus, advantages of covalent grafting must be preserved while avoiding adsorption of hydrophobic surface (that may induce enzyme denaturation).

The purposes of this chapter are: (i) synthesizing monoliths with controlled hydrophobicity, (ii) implementing those materials as supports for immobilized transaminases, (iii) performing flow transaminations on such carriers to assess a potential positive effect of surface hydrophobization.

2 Results and discussion

2.1 Hydrophobic monoliths – one-pot methylation

2.1.1 Synthesis

Hydrophobic monoliths were prepared by adding a hydrophobic agent (Methyltriethoxysilane, MTES) in the synthesis step. Several MTES proportions have been tested by adjusting the TEOS concentration: 0; 2.5; 5; 7.5; 10; 12.5 and 25 %mol of MTES as compared to the total amount of Si precursors (MTES + TEOS), kept constant. Adding MTES in the Si(HIPE) emulsion appeared to alter the morphology of the material, both at the macro and micro scales (Figure 3 - 1).

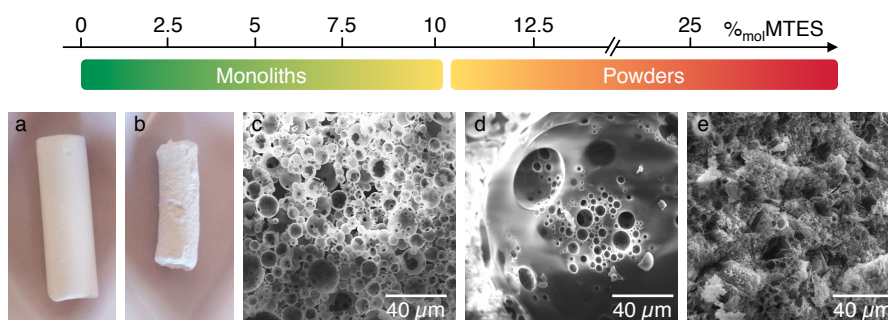


Figure 3 - 1 – Impact of MTES concentration on macroscopic aspect and on morphology of materials. (a) After drying step, Si(HIPE) is a smooth and regular-shaped monolith. (b) Monoliths containing 10 % of MTES (cast in the same mold as Si(HIPE)) undergo a non-uniform shrinkage and are irregularly-shaped. SEM micrographs: (c) Si(HIPE) exhibit a typical morphology made of hollow spherical spheres; (d) 5 % MTES monolith displays a change in morphology (uncalcined); (e) materials containing more than 10 % MTES are powders and lose their morphology (uncalcined).

Macroscopically, after the drying step, materials made by an emulsion containing up to 10 % MTES are monoliths. However, the more MTES they contain, the more the monolith shrinks during the drying. This non-uniform shrinking results in irregular-shaped monoliths (Figure 3 - 1b). Those monoliths are more fragile than

Si(HIPE) at the same stage of their synthesis (right after the drying step). 10 % MTES, materials were systematically cracked. High MTES concentrations (12.5 and 25 % MTES) invariably led to powders.

The morphology of as-synthesized materials was observed in SEM. At the scale of the macrocells, the morphology is indeed also altered by MTES addition (Figure 3 - 1). Monoliths made with 5 % MTES feature larger silica macrocells as compared to Si(HIPE). Large non-hollow silica spheres are observed and exhibit many small spherical holes. This looks like the typical morphology of a catastrophic inversion of an emulsion due to a change in the organic/water phases ratio [200]. Indeed, we have assumed that keeping the silicon concentration (TEOS + MTES) constant would result in a constant aqueous phase amount (after hydrolysis). However, due to its amphiphilic properties, MTES would not stay in the aqueous phase, but would act as a surfactant. Then, the aqueous phase amount would decrease as the MTES concentration increases. In the following, only 5 % of MTES concentration are studied and used in order to keep working with robust monolithic materials (as for Si(HIPE)).

2.1.2 Removing the organic phase

2.1.2.1 Thermal treatment

During Si(HIPE) synthesis, calcination at 650 °C is carried out after the washing step (see Experimental, Section 2.1.2.1). Calcination aims at removing the residual organic phase and strengthens the silica network. However, the calcination of one-pot methylated monoliths would undoubtedly lead to the combustion of methyl moieties. Thus, after the classical washing with THF and acetone (see Experimental, Section 2.1.2.2), we propose to apply a thermal treatment to indeed (i) remove the residual organic contaminants and (ii) strengthen the monolith. Obviously, this thermal treatment should however be performed at a temperature lower than the methyl groups decomposition temperature so that the hydrophobic agent is preserved.

First, a pristine (non-methylated) Si(HIPE) monolith was synthesized up to the drying step. This sample is labelled nc-Si(HIPE), where “nc” stands for “non-calcined”. TGA was carried out to determine the decomposition temperature of organic residues that remain in the sample after the poly-condensation, washing and drying steps (Figure 3 - 2).

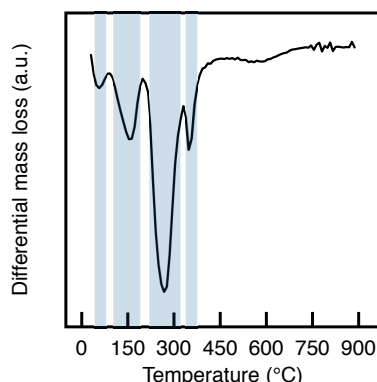


Figure 3 - 2 – dTG curve of non-calcined Si(HIPE).

Four stages of decomposition were detected (Figure 3 - 2). First, water desorbs around 50 °C. Then decomposition bands arise from 100 °C to 185 °C, 215 °C to 330 °C and 330 °C to 375 °C. They are attributed to the decomposition of organic matter (dodecane and TTAB) that was not well eliminated by the washing steps. This experiment also confirms that the classical calcination applied on the pristine Si(HIPE) supports at 650°C is appropriate to remove all organic residues.

TGA was carried out on a Si(HIPE) monolith grafted with methyl moieties (M100_D; for the nomenclature, see Experimental, Section 2.1.2.3), in order to determine the methyl combustion temperature (Figure 3 - 3). Two sets of weight loss are observed on the differential thermogravimetric (dTG) curves (Figure 3 - 3,a). First, a water desorption occurs below 80 °C (with a maximal desorption peak around 47 °C). Second, for the methylated sample, a loss is ranging from 250°C to 400 °C (shaded band on Figure 3 - 3). Because this sample only contains methyl grafted on silica, this second weight loss is assigned to the methyl decomposition. Accordingly, the calcination temperature should not exceed 250 °C if methyl groups are to be preserved. On the bare silica (Si(HIPE); Figure 3 - 3b), slight mass losses are observed from 200 to 350 °C. They are attributed to organic residues adsorbed after the calcination.

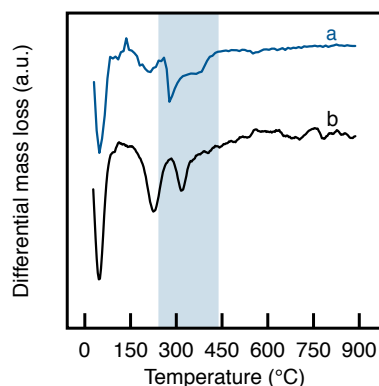


Figure 3 - 3 – Differential thermogravimetric curves (dTG). (a) Methyl-grafted Si(HIPE) (M100D) exhibits typical organic weight loss around 300 °C assigned to methyl moieties. (b) Si(HIPE) display some organic contamination (even though it has been calcined).

As the decomposition temperatures of residual organic matter on nc-Si(HIPE) (100-375 °C, Figure 3 - 2) and methyl groups (250-400 °C; Figure 3 - 3) concur, it is not possible to thoroughly eliminate the organic residues by thermal treatment while preserving the methyl moieties on the silica surface. Therefore, a deeper washing was considered to improve the removal of organic residues. A thermal treatment below 250 °C will be proceeded afterwards.

2.1.2.2 Washing the organic residues



Figure 3 - 4 – Surfactant elimination. Material is washed with aqueous acetic acid (in excess) at appropriate pH. Acetate ions electrostatically bind the surfactant that is desorbed from the surface along washings. An aqueous sodium carbonate solution is used afterwards so that carbonate ions replace acetate ions on the surface. At the end, a gentle heating under vacuum conditions allows the decarboxylation of carbonate ions (releasing carbon dioxide). Adapted from Endo *et al* [167].

A second stage of washing is implemented (after the usual washing with THF and acetone), adapted from Endo *et al* [167]. First, the solid is washed with aqueous acetic acid solution. Acetate ions are expected to be attracted towards the polar head of the surfactant (positively charged) and form an ionic bond with the surfactant, helping its desorption from the silica surface. Then acetate ions (in

excess) are adsorbed on the silica surface. The material is then washed with sodium carbonate solution to replace acetate ions. Finally, carbonate ions are removed by heating the materials at 60 °C under vacuum (Figure 3 - 4).

In practice, the pH of the acid solution must be chosen so that acetic acid solution contains significant amount of deprotonated ions (pH close or higher than $pK_A \sim 4.75$) and silica surface is not charged (to decrease interactions between surface and surfactant; pH close to isoelectric point ~ 2 [140]). So, there is no obvious ideal working window for which silica would be mostly neutral and acetic acid mostly deprotonated. However, according to the chemical equilibrium law, silica is only partially deprotonated over 2; and acetic acid is only partially protonated under pH 4.75. So, we have washed uncalcined silica monoliths at intermediate pH values (pH 2.5 and pH 4) in an attempt to benefit from the little fraction of deprotonated acetic acid and neutral silica that are still present in those conditions. TGA was carried out to assess the acid washing efficiency, with comparison to the nc-Si(HIPE) (Figure 3 - 5).

At both pH, the weight loss from 100 °C to 185 °C is avoided (shaded area on Figure 3 - 5) with the acid washing method. However, the two other peaks assigned to organic residues are still observed around 250 °C and 340 °C. Thus, it is supposed that this method did not allow to completely remove the organic residues (probably due to a lack of respectively acetate ions and neutral surface on the silica monoliths).

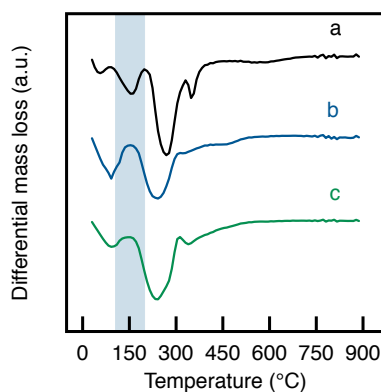


Figure 3 - 5 – dTG curves. (a) Non-calcined Si(HIPE); (b) non-calcined Si(HIPE) washed with acetic acid (pH 2.5) and sodium carbonate; (c) non-calcined Si(HIPE) washed with acetic acid (pH 4) and sodium carbonate.

Another common method for organic matter elimination consists in washing the monoliths (after the usual washing step with THF and acetone) with ethanol [166]. Non-calcined Si(HIPE) (nc-Si(HIPE)) have been immersed in ethanol for 24h and then dried (see Experimental, Section 2.1.2.2). Samples were then analysed in TGA (Figure 3 - 6).

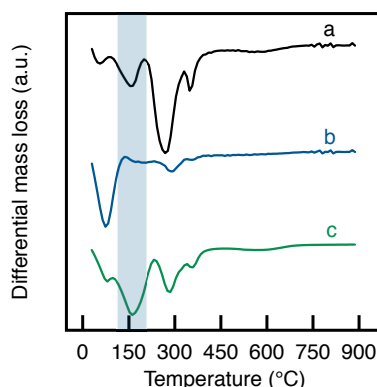


Figure 3 - 6 – dTG curves. (a) Non-calcined Si(HIPE); (b) non-calcined Si(HIPE) washed with ethanol; (c) methylated Si(HIPE) (5 % MTES in the emulsion) washed with ethanol.

Differential TGA curve of nc-Si(HIPE) washed with ethanol shows the loss assigned to water desorption (below 100 °C). Decomposition of organic matter from 225 °C to 375 °C is still observed but much less than for nc-Si(HIPE) sample that have not been washed with ethanol. This suggests that this washing method allows the elimination of large part of organic residues.

Then 5% MTES monolith prepared by the one-pot method (see Experimental, Section 2.1.2.2) was washed via the same procedure and also analysed in TGA (Figure 3 - 6). As expected, the weight loss corresponding to methyl groups is seen around 300 °C. Surprisingly, one of the organic residues peaks appears (160 °C, shaded area). It suggests that the methyl groups, due to their hydrophobic properties, located into the monoliths retain more the organic matter than the non-methylated silica.

A thermal treatment at 230 °C should eliminate that organic matter. However, after the thermal treatment at 230 °C (heating rate 1 °C /min; then plateau for 24 h) all the monoliths systematically exhibit numerous cracks.

Finally, one-pot methylated monoliths are given up due to the difficulty to produce methylated monoliths clean from other organic residues. Also their brittleness was identified as a limitation for practical application. To improve the one-pot method, one could add the MTES in the organic phase (dodecane) without changing the amount of TEOS in the aqueous phase (similarly as reported by [158] for the synthesis of hybrid aluminosilicate monoliths). The overall MTES amount could also be decreased in the material, to avoid significant impact on the monolith morphology.

In the next section, silica post functionalization by MTES is envisaged (see Experimental, Section 2.1.2.3).

2.2 Hydrophobic monoliths – Post-functionalization

Si(HIPE) monoliths are functionalized in two steps with MTES (conferring hydrophobicity) and with APTES (enabling enzyme immobilization) or vice versa.

Successive functionalizations were conducted as below: dried monoliths were first immersed in functionalization agent solution (in water saturated toluene, see Experimental, Section 2.1.2.3, for details about functionalization through the Method D). They have been then washed three times with saturated toluene. Then, instead of the drying step (as implemented in the Method D), wet monoliths were directly immersed in the second functionalization bath (containing the second functionalization agent). Then, the end of the functionalization process (washing and drying) follows the Method D.

2.2.1 Functionalization starting by APTES

In a first set of experiments, monoliths were first functionalized with APTES and then with MTES. During the second functionalization, the monoliths did not sink down into the MTES solution (unlike what was always observed for the method D functionalization), but floated on the liquid surface.

It was suggested that for APTES-functionalized monoliths, a large part of micropores were clogged by APTES. Indeed, this was confirmed by nitrogen physisorption analysis (Table 5), used to probe the textural properties of materials. No change in textural properties was observed when comparing Si(HIPE) and methylated Si(HIPE), as we grafted only few methyl moieties at the surface. In comparison, APTES grafting led to a change in textural properties (lower specific surface area, lower pore volume and pore size), attributed to a partial

pore clogging (see Chapter II). This alteration in morphology has a similar effect as a change in material polarity, as water hardly penetrates through the APTES-functionalized monoliths.

Table 5 – Textural properties of Si(HIPE), methylated Si(HIPE) and aminated Si(HIPE)

Sample	Specific surface area ($\text{m}^2\cdot\text{g}^{-1}$)	Pore volume ($\text{cm}^3\cdot\text{g}^{-1}$)	Pore size ($\text{\AA}$)
Si(HIPE)	580	0.14	28
M250 _D	590	0.14	28
A250 _D	450	0.08	23

Post grafting of A250_D monoliths was still conducted to the end (even though we can suspect a non uniform grafting, due to the fact that not all the parts of the monoliths were immersed during the second functionalization), and were tested in flow mode as enzyme carriers (*vide infra*).

2.2.2 Functionalization starting by MTES

Si(HIPE) monoliths have been sequentially functionalized first by MTES and then by APTES. In this case, the monolith could be sunk into the second functionalization batch easily (unlike the monoliths synthesized in the Section 2.2.1). Thus, macroscopically, such samples seemed to be hydrophobic enough to ensure a good compatibility with organic solvent conditions.

2.2.2.1 Characterization

TGA analyses were conducted on the monoliths after the first functionalization (either by MTES or APTES) to assess the efficiency of the organic grafting, and to observe the impact of organic moieties on water desorption (Figure 3 - 7). Indeed, the measure of wetting angle is complicated by the use of porous materials. Thus, characterizations through TGA and IR are employed to observe the water content in the materials, as a measure of hydrophobicity.

In TGA, identification of lost compounds was carried out by mass spectrometry (see Annexe 5). As a reminder, samples are labelled Ax_D and Mx_D where A stands for APTES-grafting; M stands for MTES-grafting; x is the concentration in APTES or MTES respectively in the functionalization solution; D is the method of functionalization involved.

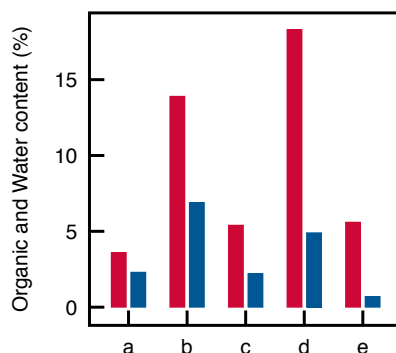


Figure 3 - 7 – Organic (red) and water (blue) content (mass percentages) assessed by TGA. (a) Si(HIPE); (b) A100_D; (c) M100_D; (d) A250_D; (e) M250_D.

Samples grafted with APTES exhibit a higher organic content, as comparison with Si(HIPE) monolith. This suggests that the functionalization method effectively allow the grafting of APTES moieties at the monolith surface (as already discussed in the Chapter I, Section 2.2). Also, the more the APTES solution is concentrated, the more the sample has a high organic content, suggesting that at 100 mM, the monolith is not saturated with APTES moieties yet. By looking at the water content of APTES-functionalized samples, it appears that samples grafted with higher APTES concentration (A250_D compared to A100_D) contain less water. This suggests that APTES moieties bring hydrophobicity to the grafted material (probably due to the propyl and ethoxy functions, and the lower porosity). Thus, APTES-grafted monoliths are slightly hydrophobic.

Organic matter is effectively grafted on the monoliths functionalized by MTES (M100_D and M250_D). By looking at the organic content (in mass), for the same molar concentrations for each organosilane in the functionalization batch, it seems that less MTES is grafted (by comparison with APTES). MTES is lighter than APTES and then we must look at the molar concentrations in the monoliths. To that end, we supposed that MTES and APTES were grafted according to a mono-dentate binding with the two other ethoxy groups being hydrolysed (Figure 3 - 8). Further considerations about the grafted structures and the hydrophobic properties are discussed in Annexe 6. MTES-grafted sample exhibit a very low water content (as compared with Si(HIPE) and APTES-grafted samples). Also, the more MTES concentration was kept high in the grafting solution, the less water they contain. This suggests that MTES samples are highly hydrophobic.

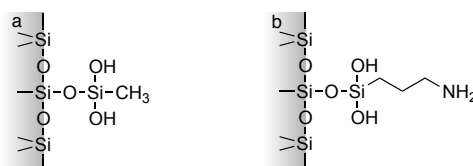


Figure 3 - 8 – Mono-dentate bounded MTES (a) and APTES (b).

In that case, grafted MTES and APTES moieties have a molar mass of respectively 76 g.mol^{-1} and 119 g.mol^{-1} . Then, by looking at the molar concentrations (Figure 3 - 9), we observed that a lower molar content of MTES molecules was indeed grafted on the monoliths. Actually, it is well known that APTES molecules exhibit a catalytic activity towards its functionalization process [140]. So we expected that more APTES moieties were grafted on the surface than MTES for the same period of time (as APTES is grafted faster than MTES due to its catalytic properties).

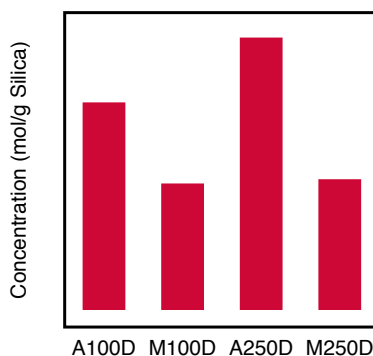


Figure 3 - 9 – Molar organic concentration of MTES and APTES functionalized samples.

Infrared analyses were conducted on pristine and functionalized samples (Figure 3 - 10, left). The pristine sample seems to be the most hydrated one as the typical water bands are relatively large (water stretching large band around 3400 cm^{-1} , and water bending band at 1640 cm^{-1}). As expected, MTES-functionalised sample has the lowest water signal (Figure 3 - 10, middle). As expected, APTES-grafted monoliths exhibit a bit more water. This is consistent with TGA analyses (see Figure 3 - 7). A typical band for C-H stretching is observed, around 3000 cm^{-1} , for all functionalized samples. An additional amine band is noticed at 1550 cm^{-1} for APTES functionalized samples.

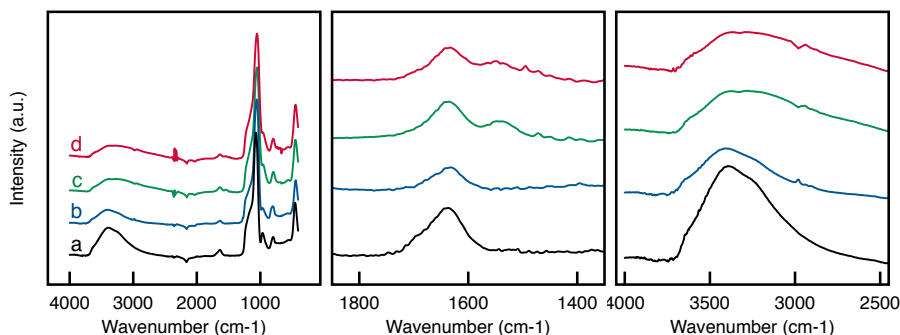


Figure 3 - 10 – Infrared spectra. (left) Overview; (middle) focus around 1600 cm^{-1} ; and (right) focus between 3000 and 3500 cm^{-1} . As expected, MTES-functionalized sample exhibits low water signal (at 1600 cm^{-1} and from 3000 to 3500 cm^{-1}). (a) Si(HIPE); (b) M250_D; (c) A250_D; (d) M250_DA250_D.

Samples that have been doubly functionalized have been tested in the catalytic reaction before characterization.

2.2.2.2 Catalytic efficiency

Functionalized monoliths have been tested as a carrier for transaminase and tested in the flow mode (see Experimental, Section 2.3.2; Figure 3 - 11). TA-M250_D-30d (transaminases have been immobilized on M250_D monolith at $30\text{ }^{\circ}\text{C}$) sample led to poor activity (1.8% yield) despite a high enzyme loading (3.7 mg). As comparison, TA-GA-A250_D-30d (as reminder, enzymes are immobilized on APTES-grafted sample, using glutaraldehyde, see Section 2.3.2) exhibits a good activity (21.5% yield, see Chapter II, Section 2.3) and a low enzyme loading (0.21 mg). Thus, it seems that hydrophobic samples allow immobilizing more enzymes due to their ability to attract hydrophobic patches located on the enzyme surface. However, this hydrophobic interaction appears to disturb the enzyme that is no more able to catalyse the transamination. This could be due to a deformation of the active-site pocket. Also, hydrophobic immobilization might lead to a more random orientation of the enzyme on the silica surface (as compared to the covalent anchoring on amino groups with is directed on the available lysine residues). So the orientation could block the entrance of the active site for some of immobilized enzymes. On the contrary, APTES immobilization forces the enzyme to be immobilized towards few directions that could be actually more favourable for the catalytic act.

Biocatalytic activity of TA-GA-A250_DM250_D-30d sample was assessed in flow (although the functionalization process was not

conducted as expected, see Section 2.2.1). Surprisingly, such biocatalyst exhibited a yield as high as the TA-GA-A250_D-30d sample. Supports functionalized with MTES and then with APTES were also tested in flow mode (TA-GA-M250_DA250_D-30d). The yield of such biocatalysts was also reaching 20 %. Thus, it is shown that functionalization sequence seems not to impact the catalytic properties. Indeed, similar activity is obtained for both TA-GA-M250_DA250_D-30d and TA-GA-A250_DM250_D-30d samples. Similar loadings were also obtained, respectively 3.2 mg and 3.0 mg per monolith. It is also suggested that MTES did not affect enzyme activities when enzymes are immobilized through covalent grafting. The presence of MTES moieties does not affect the catalytic efficiency. Most of efficient immobilization (which gave active enzymes) was probably located on APTES moieties.

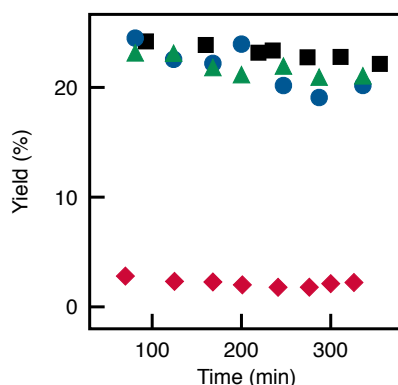


Figure 3 - 11 – Biocatalytic efficiencies. (■) TA-GA-A250_D-30d, (◆) TA-M250_D-30d, (▲) TA-GA-M250_DA250_D-30d and (●) TA-GA-A250_DM250_D-30d samples. Experimental conditions: ATA-117 (10 mg in impregnation solution), rac-BMBA (10 mM), sodium pyruvate (10 mM), PLP (0.5 g/L).

3 Conclusion

We attempt to synthesize silica monoliths with controlled hydrophobicity, in order to improve the catalytic efficiency of immobilized enzymes.

First, monoliths have been synthesized by adding the hydrophobic agent (MTES) directly in the emulsion (one-pot method). However, we showed that the synthesis was relatively difficult to control. On the one hand, monoliths showed a higher brittleness as compared with reference Si(HIPE) monoliths. On the second hand, attempts to wash the solid gel from organic residues while preserving the methyl groups were unsuccessful.

Second, Si(HIPE) monoliths were functionalized by MTES in a separate step, by post-functionalization of the preformed Si(HIPE). MTES was effectively grafted on the silica, but in very low amount. Nevertheless, methyl grafted samples exhibit hydrophobic properties as observed in IR and TGA. Methyl grafted samples also allowed to reach a much larger enzyme loading. Yet, the catalytic performance was very poor. We put forward that the hydrophobic surface of MTES-functionalized Si(HIPE) support results in a denaturation of the enzyme.

The support was also functionalized with both MTES and APTES. We showed that whatever the functionalization sequence, both led to good catalytic properties (up to 21 % yield for M250_DA250_D and for A250_DM250_D samples). However, the activity never exceeded the activity level reached with only APTES-functionalized samples (21.5 % yield). We conclude that the covalent grafting via APTES is the key to successfully immobilize the enzyme while preserving its activity and that the further addition of methyl groups does not help reaching higher performance.

Chapter IV – Production, purification and characterization of a His-tagged transaminase, and immobilization for flow transaminations

The work presented in this chapter has been carried out by Ir. Adrien Gauchet during his master thesis (2017).

1 Introduction

So far, a commercial transaminase (ATA-117) has been successfully used for flow transamination reactions. As the protein sequence is known and has been published [7], it is possible to produce the transaminase ATA-117 in-house. Doing so would open up two possibilities: (i) improving the purity of the enzyme extract to obtain a higher activity per mass of enzyme involved in the preparation of the biocatalyst monoliths, and (ii) adding a short peptide sequence (His-tag, see Introduction, Section 2.3.2.1) that can be used to anchor the enzyme in a different way. Indeed, a purer enzyme extract would allow to avoid the presence of hydrolases enzymes, that are known to destroy and inactivate enzymes. Also, a better purity would allow avoiding the potential interferences from non desirable particules located into the enzyme extract.

Transaminase immobilization through His-Tag binding have been performed by Cassimjee *et al.* [147] and Planchestainer *et al.* [71] in batch mode (see Introduction, Section 2.3.5) ; and by Matosevic *et al.* [159] and Halim *et al.* [160] in flow mode (see Introduction, Section 0).

In this chapter, we first produced and purified the His-tagged version of ATA-117 (His-TA). To do so, a plasmid (see Annexe 7) containing the gene coding for ATA-117 with a His-tagged termination was purchased and transformed in *E. coli*, and the enzyme was expressed and purified. This part of the work has been conducted in the biophysics, biochemistry and microorganism genetics laboratory (UCLouvain, Belgium) under the supervision of Prof. Patrice Soumilion.

Then, the purified enzymes were immobilized on grafted silica monoliths prior to flow transaminations (in the kinetic resolution mode). For that purpose, two immobilization methods have been studied: (i) Epoxy-grafted silica monoliths activated with lysine-nitrilotriacetic acid loaded with nickel atoms (Lys-NTA:Ni) have been

used for immobilizing His-TA through coordination bond with the His-tag (as suggested by Liu *et al.* [201]). GLYMO (see Experimental, Section 2.3.2.2 and Figure E - 8b) was used as a functionalisation agent to graft the silica monoliths with epoxy functions. (ii) APTES-grafted silica monoliths were activated with glutaraldehyde prior to His-TA immobilization, taking advantage of the presence of lysine groups at the enzyme surface. This is the same method as for previous chapters (Method D, see Experimental, Section 2.1.2.3).

Grafted carriers have been characterized (by IR, TGA, SEM, ICP-AES). Biocatalysts have been tested in flow mode, in the kinetic resolution mode.

2 Results and discussion

2.1 Enzyme production, purification and characterization

Three successive protein expressions (see Experimental, Section 2.1.1) have been conducted. From each expression-purification step, we obtain 4 mL aliquots enzyme solution. Concentration of each aliquot, namely Lot2, Lot3 and Lot4, was respectively 10.85, 14.06 and 14.18 g/L (Lot1 was lost due to a bad manipulation). Thus, at the end of the purification steps, we obtained roughly 150 mg His-TA.

SDS-PAGE has been done on His-TA and Codexis ATA-117 transaminases to verify the presence of the enzyme in the extract and to obtain a qualitative information on its purity (Figure 4 - 1). A large band (black arrow) was detected in most wells between the bands assigned to 35 kDa and 40 kDa. This is assigned to the transaminase which is known to have a molecular weight of 38.48 kDa. Wells 3 and 4 seemed empty as no band is observed in the area of interest. It is suspected that an error of manipulation occurred. Codexis enzyme has been inserted in wells 9 and 10 (Figure 4 - 1). The band at ~37 kDa was barely visible for this sample, suggesting that the purity of the commercial ATA-117 stock was relatively low. Those results suggest that the His-TA was produced in high concentration and with high purity (purity is much higher than Codexis ATA-117 stock). Again, when gram of Codexis ATA-117 enzyme is mentioned, the reader must understand gram of lyophilized powder.

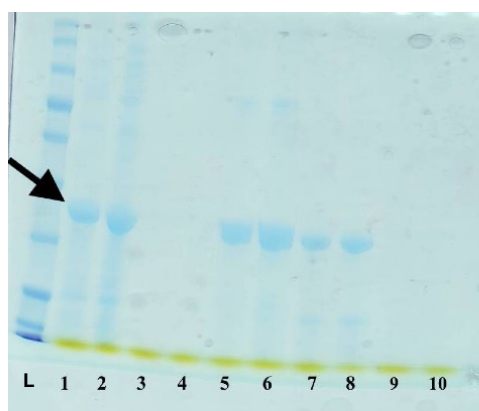


Figure 4 - 1 – SDS-PAGE Gel. Well L is the molecular weight reference (PageRuler). Lots 2, 3 and 4 were respectively inserted in wells 1 to 4, 5 and 6, and 7 and 8. Codexis enzyme has been inserted in wells 9 and 10. Odd well numbers contained 5 μg enzyme (or powder in the case of Codexis enzyme); even well numbers contained 10 μg enzyme (or powder). Wells 1 and 2 contained Lot2 enzyme, but aged during 135 days. Wells 3 and 4 (Lot2, fresh) apparently suffered from an error of manipulation as no band is observed.

2.2 Enzyme activity

2.2.1 Free enzyme activity

The lots of His-TA have been tested in batch mode with enzyme concentration of 0.05 g/L in each reactor (Figure 4 - 2). Then, k_{OBS} have been calculated (Table 6).

Table 6 – Catalytic efficiency of His-TA and ATA-117 transaminases (for the calculations, see Annexe 1.1).

Transaminase lot	k_{OBS} (min^{-1})
Blank	0.001
His-TA lot 2	0.093
His-TA lot 3	0.100
His-TA lot 4	0.108
ATA-117 (Codexis)	0.023

As expected, His-TA samples are more active than the Codexis ATA-117 stock. It is explained by the difference of purity (as Codexis ATA-117 is less pure than His-TA aliquots). From those results, it is suggested that the His-TA produced in our laboratory is approximately 4 times more pure than the Codexis TA. His-TA lots

have similar relative activities. Catalytic efficiency (see Annexe 1.1) of His-TA, k_{CAT}/K_m , reaches $7.4 \cdot 10^4 \text{M}^{-1} \cdot \text{min}^{-1}$, and is then 10 times higher than for Codexis ATA-117. Enantioselectivity was confirmed by chiral-HPLC. Racemic mixture is observed at the beginning of the reaction. R-BMBA is gradually transformed into BAP. Enantiomeric excess reaches more than 99 % (no residual R-BMBA is observed when the global yield reaches 50 %).

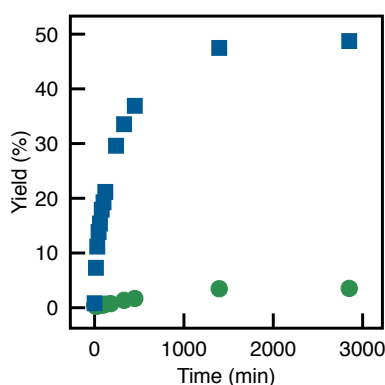


Figure 4 - 2 – Evolution of the BAP yield as a function of time in a batch reaction with the free His-TA enzyme produced in our laboratory (■, Lot2). BAP yield reaches its maximum (50 % for kinetic resolution). The three different His-TA lots gave similar results (not shown). (●) Green dots are the blank (no enzyme). Experimental conditions : His-TA (0.05 g/L), rac-BMBA (10 mM), sodium pyruvate (10 mM), PLP (0.5 g/L).

2.2.2 His-tagged Immobilized enzyme activity (in batch mode)

His-TA has been immobilized on commercial NTA:Ni agarose beads in order to check how immobilization could affect its activity. 2 mL beads have been rinsed with 30 mL phosphate buffer and then inserted into 5 mL His-TA solution (0.05 g/L), under mild stirring at 4 °C for 90 minutes. The beads have then been filtered, rinsed (30 mL phosphate buffer, 0.1 M, pH 8), and stored into a vial containing sodium pyruvate (10 mM) and PLP (0.5 g/L) in the phosphate buffer. Beads have then been used in batch mode (after filtration) using the method depicted in Experimental, Section 2.3.1.

When immobilized, His-TA is still active. However, catalytic efficiency (k_{OBS}) of immobilized His-TA is lower (0.15 min^{-1}) that is only 15 % of free His-TA activity in the same conditions. It should be mentioned that 0.25 mg His-TA have been inserted in batch reactor in both cases (we hypothesized that all enzymes in contact with the NTA:Ni beads have been immobilized on it, as the maximum loading

capacity of beads is 117 mg/mL). Thus, a significant enzyme deactivation occurs when His-TA is immobilized through their His-tag (that is often expected when enzymes are immobilized, due to a change in the enzyme environment, see Introduction, Section 2.3.2.1). However, this deactivation is not restrictive for the following as the activity when immobilized is still quite high (and largely quantifiable).

2.3 GLYMO-grafted monoliths characterization

It must be noted that the grafting method used in this chapter for GLYMO-functionalization was the Method B (see Experimental, Section 2.1.2.3) and not the fully optimized Method D because, chronologically in the course of this project, the grafting method optimization (see Chapter II) has been done in parallel with the study of the His-TA performance. As a reminder, Method B allowed to ensure a good dispersion of the functionalization agent in the case of APTES grafting, yet still suffering from a certain lack of reproducibility. It is hypothesized that the silanization process using GLYMO proceeds in a similar manner. Therefore the results presented below could potentially be improved further by applying the fully optimized Method D for the functionalisation.

GLYMO-grafted monoliths have been analysed by ATR-FTIR (Figure 4 - 3, left). The peak assigned to C-H vibration (between 2900 and 3000 cm^{-1} ; Figure 4 - 3, Middle) is observed for GLYMO-functionalized samples and is assigned to the presence of organic matter on the grafted silica monoliths [202]. Indeed, GLYMO functions enclose a propyl group. This peak is not observed for the pristine Si(HIPE). Moreover, the peak intensity assigned to GLYMO-grafted samples is increasing with the GLYMO concentration (for G100_B and G200_B samples). It remains stable upon further increasing the GLYMO concentration (G500_B). This indicates that beyond 200 mM GLYMO in the grafting solution, the silica monoliths seemed to be saturated in GLYMO.

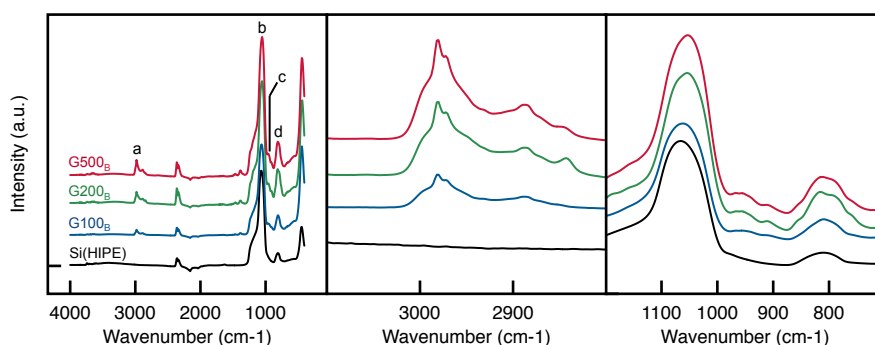


Figure 4 - 3 – ATR-FTIR spectra. (Left) Overview. GLYMO-functionalized samples feature a characteristic peak between 2900 and 3000 cm^{-1} assigned to C-H vibration (a). The peaks assigned to lattice vibration (b) and silanols (d) partially hide the peak assigned to epoxide groups (c). (Middle) Focus on 3000 cm^{-1} area ; (right) Focus on 1000 cm^{-1} area.

An absorbance band (between 810 and 950 cm^{-1} ; Figure 4 - 3, Right) assigned to epoxide functions [202] should have been observed for GLYMO-grafted samples. However, as this band would be located between two larger bands assigned respectively to silanols vibration (around 800 cm^{-1}) and lattice vibration (around 1100 cm^{-1}), only a slight shoulder is observed.

GLYMO grafted monoliths have also been analysed through epoxide titration (see Experimental, Section 2.2.8) in order to quantify the epoxide content (Figure 4 - 4). GLYMO concentration in the grafted monoliths (expressed as relative GLYMO content, *i.e.* the amount of GLYMO functions, in μmol , in one gram of monolith) is increasing as the GLYMO concentration in the grafting solution increases. The relation confirms the results observed in ATR-FTIR. However, unlike what was observed in ATR-FTIR, no saturation is observed beyond 200 mM. We must mention that, during the titration, the pH of the solution was very low (measured at 1) and could induce a partial silica dissolution, that could explain the value obtained without epoxide)

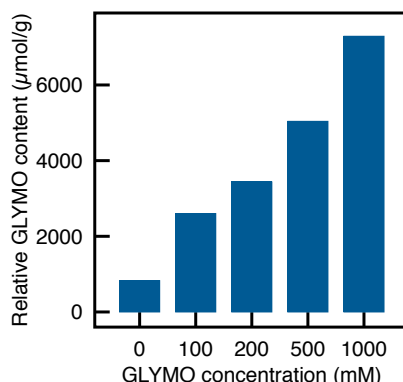


Figure 4 - 4 – GLYMO concentration determination. Relative GLYMO content in grafted monoliths ($\mu\text{mol GLYMO} / \text{g monolith}$) versus GLYMO concentration in the grafting solution (mM).

Thermogravimetric analyses have been carried out on a GLYMO-grafted sample (G1000_B) and on the pristine Si(HIPE) monolith (Figure 4 - 5). The samples have been dried at 105 °C for 24 h under vacuum prior to analysis. A large mass loss is observed for G1000_B sample, and is assigned to organic matter decomposition (brought by GLYMO). The final mass reaches 55 % of the initial mass. It results that the GLYMO content in monolith is 3157 $\mu\text{mol/g}$ (per gram of monolith). This value is 2.3 times lower than the relative GLYMO content measured through epoxide titration, but still roughly in the same order of magnitude.

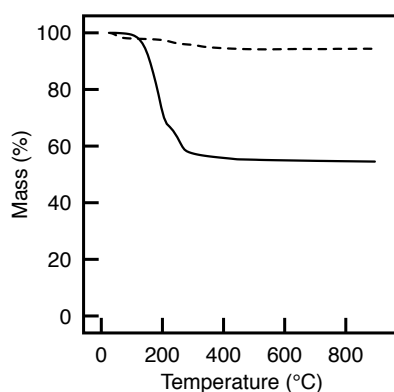


Figure 4 - 5 – TGA of GLYMO-grafted sample. G1000_B (solid curve) and Si(HIPE) (dotted curve).

2.4 Flow transamination

For the next set of experiments, loading evaluation has been conducted by a mass balance, using residual activities (see Experimental, Section 2.3.2.3). However, those measurements suffered from high variability. For some experiments, no loading values could be calculated. We suspected that the enzyme concentrations measured were too low to get an accurate loading value. Nonetheless, loading values were systematically below 1 mg.

Detailed reaction conditions and results are given in Annexe 8.

2.4.1 Role of epoxide and NTA:Ni functions at the monolith surface

Flow reactions were conducted on monoliths prepared by the impregnation of His-TA on a pristine Si(HIPE), GLYMO-grafted Si(HIPE) and GLYMO-grafted Si(HIPE) activated with Lys-NTA and nickel chloride (Figure 4 - 6, and see Figure E - 8 for global scheme). In that way, it was first possible to identify the role of epoxide functions on the enzyme immobilization. In fact, epoxide groups could link the aminated residues located at the enzyme surface (see Introduction) and therefore participate in an unspecific enzyme immobilization that does not involve the His-tag. To achieve the specific anchorage of the enzyme via the His-tag, the GLYMO functionalized monoliths first have to be incubated with NTA:Ni.

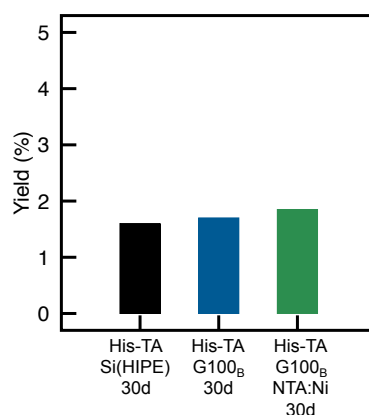


Figure 4 - 6 – BAP yield for His-TA immobilized on Si(HIPE), GLYMO-grafted monolith and GLYMO-grafted monolith activated by Lys-NTA and nickel chloride. As a reminder, « 30d » stands for enzyme immobilization at 30 °C.

The presence of GLYMO and NTA:Ni slightly increased the yields of the flow reactions, as compared to the simple adsorption on a pristine Si(HIPE) or to the GLYMO functionalized monolith in the

absence of NTA:Ni. However, the yields of the reactions for those biocatalysts (His-TA-Si(HIPE)-30d, His-TA-G100_B-30d and His-TA-G100_B-NTA:Ni-30d) remain very low (respectively 1.6 %, 1.7 % and 1.85 %). Flow reaction using biocatalysts containing more GLYMO (His-TA-G200_B-NTA:Ni-30d, Figure 4 - 7) led to a very small increase in the yield (2 %). At this stage, it seems that the strategy of immobilization via the His-tag was not successful, both in terms of enzyme loading and in terms of catalytic performance.

2.4.2 Role of nickel atoms on the His-tag immobilization

We suspected that the low yield obtained above could be due to a low nickel loading on the monoliths. Indeed, the Lys-NTA and NiCl₂ solutions passed through the monolith with a low residence time (around 42 seconds). We then proposed to fill the reactor with Lys-TA or nickel chloride solutions and then stop the syringe pump so that the reactor is let to rest for a given period (2 or 14 h). After this incubation period, the reactor is washed prior to enzyme immobilization (as reported in Experimental, Section 2.3.2.2; Figure 4 - 7).

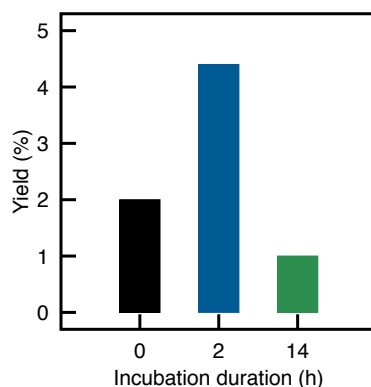


Figure 4 - 7 – Effect of Lys-NTA and NiCl₂ incubation duration on BAP yield. Biocatalysts are His-TA-G200_B-NTA:Ni-30d, His-TA-G200_B-NTA2h:Ni2h-30d and His-TA-G200_B-NTA14h:Ni14h-30d.

A slight increase in the yield (4.4 %) was observed for biocatalysts incubated 2h with Lys-NTA and 2 h with nickel chloride solutions (His-TA-G200_B-NTA2h:Ni2h-30d). However, this trend is not observed for the long incubation time (His-TA-G200_B-NTA14h:Ni14h-30d) as the yield was only 1.8 % for this biocatalyst. It is suspected that high nickel content (obtained by long incubation time) could be harmful for transaminases.

An attempt for immobilizing His-tagged enzymes on another metal was conducted. As nickel, cobalt is widely used in enzyme purification for its ability to form complexes with His-tag [71]. Cobalt ions exhibit a high selectivity for His-tagged enzymes (allowing the production of highly pure enzyme, when Co is used in enzyme purification columns). However, the affinity between Co and His-tag is lower the affinity between Ni and His-tag. Thus, cobalt based carriers exhibit a lower binding capacity [203].

Then, we conducted flow reaction using biocatalyst treated with cobalt nitrate solution instead of the Ni chloride solution (Figure 4 - 8). The BAP yield was higher (up to 7 %) than experiments using nickel atoms (2 %). However, the yield was very instable and seemed to decrease over time. It is suspected that a slow enzyme leaching occurred over time, due to the lower affinity between cobalt ions and His-tagged enzymes.

As the yields remain low, it is suspected that monoliths hold very low metal ions. Metal quantification was conducted by ICP-AES to find out the metal concentration on Ni and Co grafted monolith, and to identify a possible difference in metal affinity for NTA moieties. ICP-AES analyses have been conducted on metal grafted monoliths (G200_B-NTA:Ni and G200_B-NTA:Co). It resulted that G200_B-NTA:Ni monoliths contains more metal than the cobalt-grafted monoliths (respectively 980.5 mg_{Ni}/kg and 565 mg_{Co}/kg). For comparison, the commercial nickel-grafted agarose beads contain 23 987 mg_{Ni}/kg. Compared to the amount of epoxide grafted on the monolith (measured by titration), G200_B-NTA:Ni and G200_B-NTA:Co have respectively 0.4 % and 0.27 % epoxides involved in metal immobilization, which is low. This confirms that each monoliths bare few metal ions on its surface, and that G200_B-NTA monoliths hold more nickel atoms than cobalt atoms (probably due to a better affinity for NTA from the nickel atoms). Also, the low metal content could suggest that few Lys-NTA have been grafted on the epoxi-monolith. One could increase the Lys-NTA concentration during the impregnation of the monolith, to obtain more NTA at the surface. However, even if there were low metal grafted on the surface, immobilized enzymes are still active. This suggests that the His-tag bonding is efficient (at least for G200_B-NTA:Ni) and enzymes are not denaturated through this link.

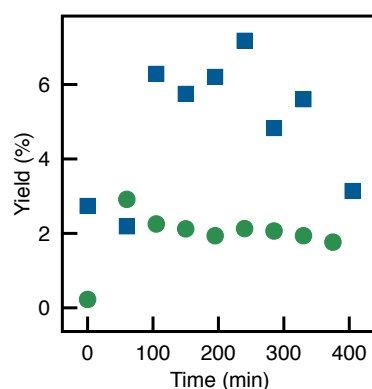


Figure 4 - 8 – Effect of metal on BAP yield. (●) Monolith impregnated with nickel (G200_B-NTA:Ni) and (■) monolith impregnated with cobalt (G200_B-NTA:Co).

2.4.3 Role of His-tag moiety on transaminase immobilization

Flow transaminations have been conducted using His-TA and Codexis ATA-117 transaminases immobilized on G500_B activated with Lys-NTA and NiCl₂. Codexis ATA-117 does not contain any His-tag.

The BAP yield was a bit higher for the reaction catalysed with the His-TA as compared to the Codexis enzyme (2.5 % versus 1.7 %). Indeed, His-TA transaminase contains a His-tag moiety that allows the enzyme to be immobilized through the His-tag method. The Codexis ATA-117 transaminase is also immobilized on the surface (as we observed stable activity over time), but we suspect it is immobilized through epoxide-amine bond (suggesting that not all epoxy moieties are activated with Lys-NTA :Ni). The difference in activity between the two catalysts could be due to (i) either the His-tag binding is more efficient than the epoxide-amine bond, and then, more enzyme stay on the surface after washing steps, leading to higher activity (due to the more enzyme grafted) ; (ii) or, the ATA-117 transaminase is less efficient than His-TA, or ATA-117 is present in lower proportion (due to its low purity).

2.4.4 Immobilization of purified transaminase on APTES-functionalized monolith

We have shown that His-tagged version of ATA-117 (His-TA) was highly pure as compared with its commercial non-His-tagged version (Codexis ATA-117, see Section 2.1). Since the grafting via the His-tag gave disappointing results, we proposed to immobilize the His-TA

on APTES-grafted monolith (A250_D), using the optimized method (see Experimental, Section 2.3.2.2, and Figure 4 - 9).

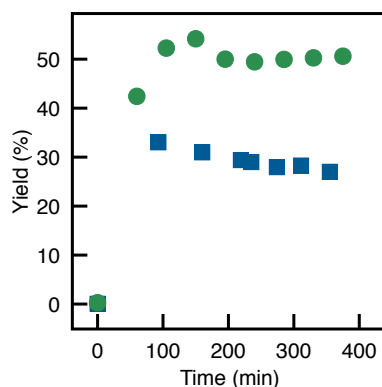


Figure 4 - 9 – Biocatalytic efficiencies. BAP yield for (●) His-TA immobilized on APTES grafted monolith (His-TA-GA-A250_D-30d) as compared with (■) Codexis ATA-117 immobilized on the same support (TA-GA-A250_D-30d).

His-TA-GA-A250_D-30d biocatalyst was highly active as the maximum theoretical BAP yield (50 %) was obtained for a contact time of 10 min. This level of activity is measured after 105 min time on stream which is roughly the time needed for the fresh solution to reach the collection vial after passing through the reactor. As comparison, the same experiment using Codexis ATA-117 (TA-GA-A250_D-30d) led to 27 % yield. Those results suggest that the high purity of His-TA allows immobilizing proportionally more transaminase, leading to more efficient biocatalyst. We must also note that the time-stability of immobilized His-TA is better than for Codexis ATA-117.

Enantiomeric excess of His-TA-GA-A250_D-30d outflows has been assessed by chiral HPLC. Only the S-BMBA enantiomer was detected, suggesting that the immobilized His-TA was still totally R-enantioselective.

3 Conclusion

We have successfully produced a his-tagged version of ATA-117 transaminase with high purity. His-TA is active in batch and flow mode.

Characterizations showed that GLYMO-grafted monoliths were effectively functionalized with epoxy-functions. However, only low

nickel (or cobalt) loadings were obtained. That could explain the low yield obtained for the reactions catalysed by the monoliths prepared by grafting the enzyme via the His-tag (maximum yield was 4.4 %).

The flow transamination catalysed by His-TA immobilized on APTES-grafted monoliths (His-TA-GA-A250_D-30d) provided excellent yield (50 %, *i.e.* the maximum for kinetic resolution) and enantioselectivity (> 99 %). Measurement have been conducted at a contact time of 10 min (standard conditions). Experiments involving shorter contact time should be carried out to identify the contact time that allows working in initial activities.

Thus, the His-tag did not help for the enzyme immobilization (as GLYMO-Lys-NTA :Ni carrier did not allow to get satisfying biocatalysts). However, the His-tag allows to produce more pure enzyme and thus, increases the overall efficiency.

Chapter V – Driving equilibrium towards the asymmetric synthesis of chiral amines

1 Introduction

In previous chapters, we have developed efficient biocatalyst for flow transamination (TA-A250_D-GA-30d, see Chapter II, Section 2.3). We demonstrated that ATA-117 transaminase could be efficiently immobilized on amino-grafted Si(HIPE) monolith and was active enough (27.5 % yield, obtained with 10 minutes contact time, see Chapter II, Section 2.3) when immobilized.

So far we have processed transamination reactions in the kinetic resolution way, which is convenient to validate that the enzyme was active when immobilized. However, in an applied perspective, the asymmetric synthesis, aiming at the production of relevant chiral amines, is probably the most relevant process. Asymmetric synthesis, however, is thermodynamically disfavoured. Therefore, it requires driving the equilibrium, e.g. through product elimination (see Introduction, Section 2.2.4).

In this chapter, several strategies are considered to drive the equilibrium towards the production of R-BMBA (for enhancement strategies, see Introduction, Section 2.2.4). They are first optimized in batch mode. Then, the most promising system is tentatively applied to the flow asymmetric synthesis.

2 Results and Discussion

Reaction conditions from this section are specified in Annexe 0.

As expected (see Introduction, Section 2.2.3), performing the asymmetric synthesis of BAP, without any equilibrium displacement strategies, led to poor activity (Figure 5 - 1).

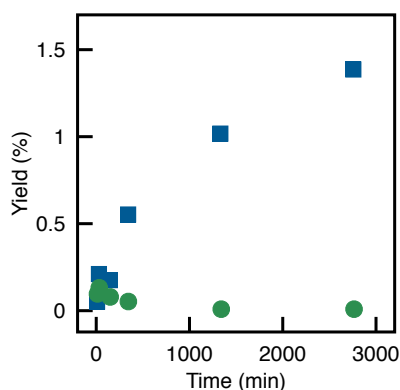


Figure 5 - 1 – Yield over time in batch mode. Initial conditions were : BAP 10 mM, isopropylamine 10 mM, PLP 0.5 g/L, ATA-117 1 g/L, DMSO 5 %, phosphate buffer 100 mM, pH 8, 30 °C. (■) ATA-117 ; (●) no enzyme.

2.1 Mono-enzymatic systems (in batch mode)

In this section, we first studied systems that involved only one enzyme, the transaminase. The equilibrium displacement is guaranteed by the transformation of one of the transamination product (the keto by-product), respectively through product polymerization and precipitation, or through product decomposition. Then, according to Le Chatelier's principle, the equilibrium is displaced.

This mono-enzymatic method is less expensive than the multi-enzymatic ones, as only one enzyme should be provided.

2.1.1 Equilibrium displacement towards product precipitation

In this system, proposed by Green *et al.* [79] (Figure 5 - 2, and see Introduction, Section 2.2.4.2), the amino-donor is o-xylylenediamine (XDD). The transamination allows the production of a targeted chiral amine, together with 2-formylbenzylamine (FBA), which spontaneously tautomerizes into 1H-isoindole. Then, 1H-isoindole polymerizes into a blue compound. Thus, as one of the

products of the transamination is consumed (by the tautomerization and polymerization reactions), the equilibrium is displaced towards the asymmetric synthesis. Green *et al.* successfully performed the amination of aromatic amino-acceptors with high yield (99 %) and enantiomeric excess (≥ 99 %).

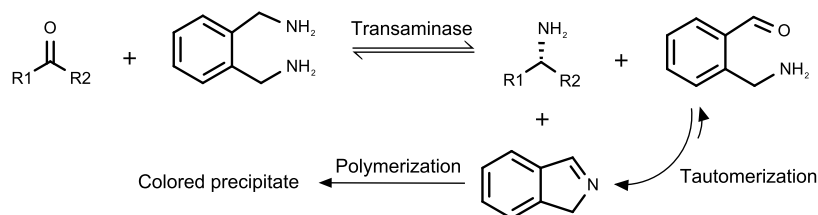


Figure 5 - 2 – Mono-enzymatic model (product precipitation). The reaction between amino-acceptor and o-xylylenediamine (XDD) aims at the production of an unstable transaminated product (FBA), which spontaneously tautomerizes and polymerizes into a blue compound. This product consumption drives the transamination equilibrium towards the asymmetric synthesis way.

In a first attempt, the enantioselective production of R-BMBA was targeted, using BAP as an amino-acceptor and ATA-117 as the enzyme. No BMBA was detected over time (Figure 5 - 3), and no change in colour was observed even after 24 h reaction. We first suspected ATA-117, to not be able to accept XDD in its active site. Then, after some discussions with the authors, we were advised to try the same experiment with ATA-256, (one of the transaminases used by Green *et al.* , see [79], also developed by Codexis.) an enantioselective transaminase. A low production of BMBA was observed over time (Figure 5 - 3).

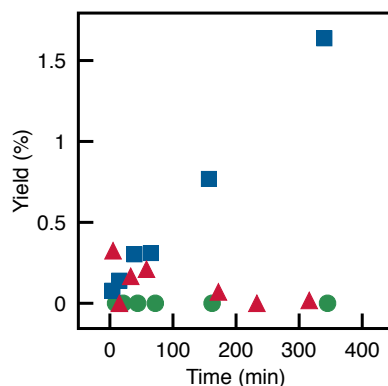


Figure 5 - 3 – Mono-enzymatic model. Yield through reaction between BAP and XDD, respectively catalysed by (▲) ATA-117 and (■) ATA-256. (●) Green dots represent the blank test (no enzyme).

To further increase the activity, we were suggested to use another amino-acceptor, the (4-fluorophenyl)acetone (FPA; Figure 5 - 4, Right; detected through gas chromatography, see Experimental, Section 2.3.1.4). Satisfying conversion of FPA was observed over time (Figure 5 - 4, Left). An oily phase was systematically observed in the batch reactors as the reaction occurred. Bi-phasic systems are interesting to implement in flow mode from a practical point of view, but are out of the scope of this project.

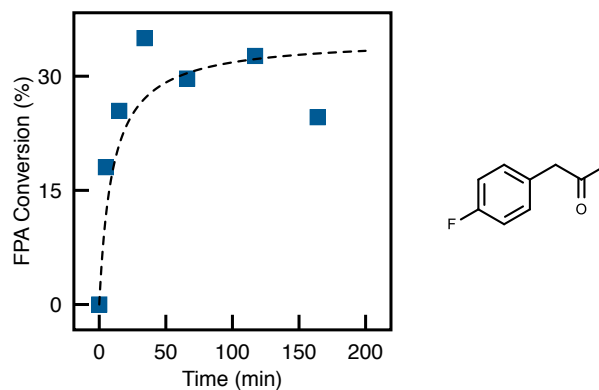


Figure 5 - 4 – Mono-enzymatic model, using FPA as substrate. (Left) FPA conversion catalysed by ATA-256 in the presence of XDD. (Right) 4-(Fluorophenyl)acetone (FPA) structure. Dotted line acts as a guide to the eye.

An attempt to catalyse the reaction between XDD and FPA was carried out with ATA-117. However, no conversion occurred, confirming that ATA-117 could not accept XDD as an amino-donor

(see Annexe 10). A larger concentration of organic co-solvent (e.g. DMSO) could be considered to avoid the formation of bi-phasic medium. But in order to keep our initial co-solvent concentration, we focussed on other strategies to shift the equilibrium towards the asymmetric synthesis.

2.1.2 Equilibrium displacement towards product decomposition

The strategy proposed by Green *et al.*, based on FBA tautomerization and polymerization, prompted us to consider a keto-product that would spontaneously decompose to shift the equilibrium in a similar way. To that end, we processed the reaction between BAP and DL-3-aminobutyric acid (Figure 5 - 5). The reaction leads to the production of acetoacetic acid (AAA), which can undergo decomposition into acetone and CO₂ [204].

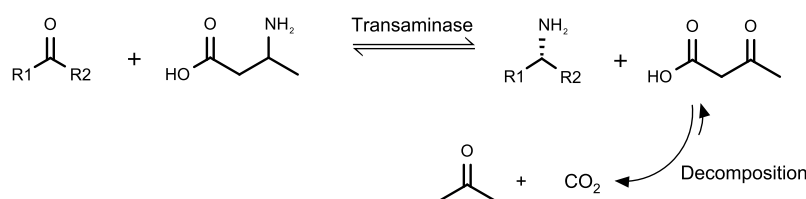


Figure 5 - 5 – Mono-enzymatic model (product decomposition). Synthesis of a chiral amine, starting from a pro-chiral amino-acceptor (e.g. BAP) and 3-DL-aminobutyric acid. The concomitant production of an unstable transaminated product, acetoacetic acid (AAA), would spontaneously decompose into acetone and carbon dioxide. As one of the products of the transamination would be consumed, the transamination equilibrium would be driven towards the asymmetric synthesis.

This reaction has been slowly catalysed by ATA-117 as a low yield was observed over time (Figure 5 - 6). However, this reaction was too slow to be applied on flow experiment. In fact, one needs to implement a catalyst to speed up the AAA decomposition [205]. For instance, AAA decomposition can be catalysed by methylamine, or derivatives, used as a homogeneous catalyst [206] or by acetoacetate decarboxylase enzyme [207]. Moreover, the production of carbon dioxide could lead to an undesirable acidification of the system (due to the solubilisation of CO₂ in aqueous medium), and to a production of gaseous compound in the middle (that is not advisable for flow systems). Thus, we decided to give up this system (that in addition could require a substantial study on such catalysts that is out of the scope of this project).

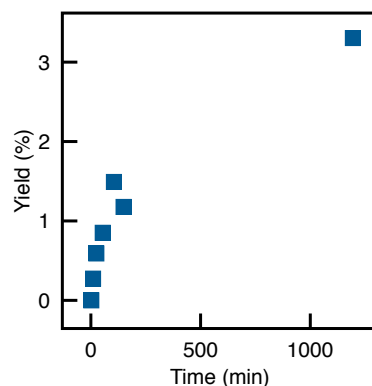


Figure 5 - 6 – Mono-enzymatic model. R-BMBA production catalysed by ATA-117 in the presence of 3-DL-aminobutyric acid.

2.2 Multi-enzymatic systems (in batch mode)

The implementation of multi-enzymatic systems is currently of increasing interest (in batch mode) [85,86,208–210]. In such systems, several enzymes are used for driving the equilibrium towards the asymmetric synthesis of chiral amines. For example, one is catalysing the consumption of transamination by-product, using a cofactor (typically the expensive NADH); the other one is catalysing the recycling of the cofactor, allowing decreasing the cost of the global process. The main challenge is to find a common working window for all enzymes. As a reminder, experimental conditions are presented in Annexe 0.

In a first attempt, we worked with the system inspired from Cassimjee *et al.* [85]: Transaminase (ATA-117) – Yeast alcohol dehydrogenase (YADH) – Glucose dehydrogenase (GDH) (Figure 5 - 7).

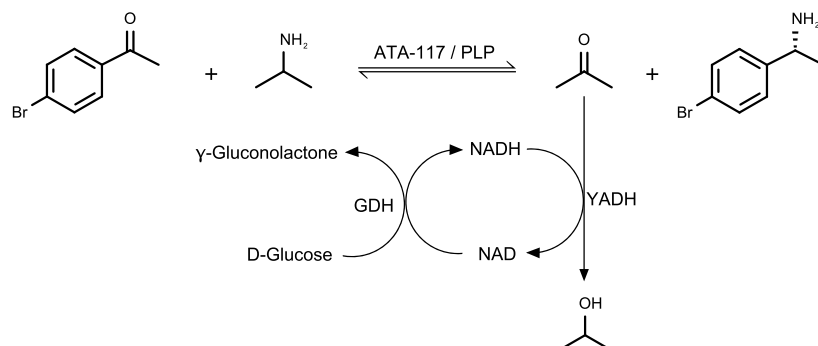


Figure 5 - 7 – Multi-enzymatic model (ATA/YADH/GDH). This reaction involves BAP as the amino-acceptor, and isopropylamine as the amino-donor. The ATA-117 catalysed reaction allows the production of R-BMBA and acetone. The latter co-product is then transformed into isopropyl alcohol by the YADH enzyme, using NADH as a cofactor. The NADH is recycled by the glucose oxidation reaction catalysed by the GDH enzyme.

Using this system, the yield of R-BMBA production was very low (Figure 5 - 8, left). However, we were using a low substrate concentration (to keep the concentration range that was working successfully in the kinetic resolution way, see Chapter II; *i.e.* 10 mM for both isopropylamine and BAP) unlike Cassimjee *et al.* (they were using 750 mM isopropylamine). We have then tried to boost the activity by increasing amino-donor, enzymes, and co-reactants concentrations (Figure 5 - 8, right). A slightly better yield (around 2 % after 1300 minutes reaction) was observed, but still too weak for flow application. In addition, we have measured a high pH at the end of the reaction (around 10). We suspect that the high isopropylamine concentration, a basic compound, was harmful for the multi-enzymatic system.

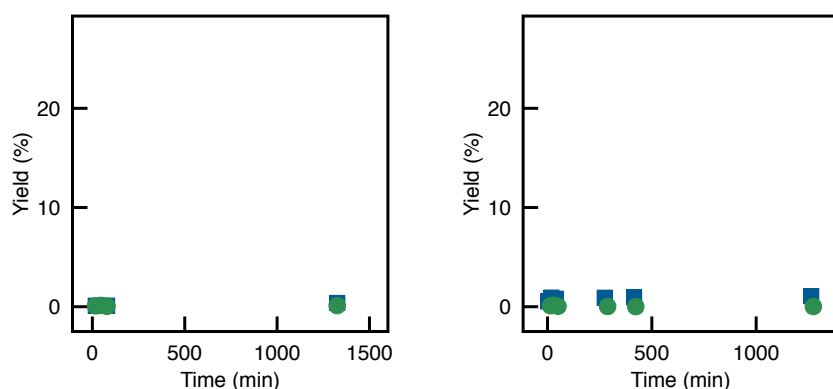


Figure 5 - 8 – ATA-117 – YADH – GDH system. (left) First attempt; (right) after boosting the activity by increasing amino-donor, enzymes and co-reactants concentration (right) In both cases, (■) blue squares represent the enzyme-catalysed reaction, green dots (●) are the blank tests (no enzyme).

So, a similar experiment was conducted, but before enzymes addition in the reactor, concentrated phosphoric acid solution was added to the reaction medium in order to decrease the pH down to pH 8 (counteracting the alkaline effect of isopropylamine; Figure 5 - 9). In those conditions, we obtained satisfying yield over time.

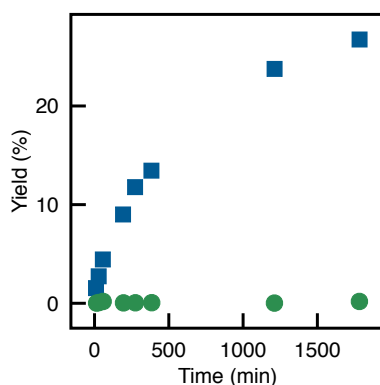


Figure 5 - 9 – ATA-117 – YADH – GDH system activity. pH neutralization prior to enzymatic reaction. Blue squares (■) represent the enzyme-catalysed reaction; green dots (●) are the blank tests (no enzyme).

Further optimization of the system was attempted, prior to the application to flow transformation. NADH concentration has been adapted to the optimized concentration tolerated by YADH enzyme (see Annexe 12). NAD was added in order to ensure that GDH activity was not limited by its cofactor concentration at the beginning

of the multi-enzymatic reactions. Also, we have tried to not use high isopropylamine concentration in order to avoid the addition of concentrated phosphoric acid solution. However, those conditions led to a poor activity (Figure 5 - 10, left). Then, we decided to keep the high isopropylamine concentration (and neutralisation by phosphoric acid). Also, we have adapted the enzymes concentrations to the specific k_{OBS} evaluated (respectively 0.023 min^{-1} , 0.0028 min^{-1} and 0.15 min^{-1} for ATA-117, YADH and GDH, see Annexe 1.1 for calculations; *i.e.* a ratio of 6:60:1 respectively). Practically speaking, the enzymes were then introduced at different concentrations, *i.e.* 0.1, 1 and 0.0167 g/L for ATA-117, YADH and GDH respectively. This led to satisfying activity (Figure 5 - 10, right). Then, we still tried to increase the activity for an efficient application in flow mode. Indeed, we need a high yield after few minutes (in our flow system, the contact time is 10 minutes). At this stage, we suspected the low efficiency of the multi-enzymatic system was due to the slow activity of YADH (~ 10 times lower than ATA-117, and consequently, put in 10 times higher concentration to respect the 6:60:1 ratio). Attempt for increasing its activity was conducted, using ethylamine as amino-donor (instead of isopropylamine). Then, the transamination step would lead to the production of acetaldehyde, which is a more natural substrate for YADH [211,212]. However, experiments conducted with such amino-donor were not conclusive (yield was lower than in the isopropylamine-system, see Annexe 13).

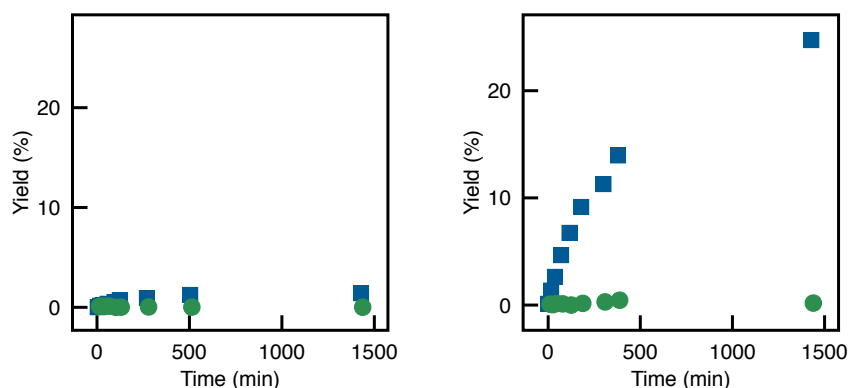


Figure 5 - 10 – Multi-enzymatic model (ATA/YADH/GDH). (left) Low isopropylamine concentration led to poor activity. (Right) Yield of the multi-enzymatic system, by adapting enzyme concentration to specific k_{OBS} (6:60:1 ratio for ATA:YADH:GDH concentrations). In both cases, blue squares (■) represent the enzyme-catalysed reactions, green dots (●) are the blank tests (no enzyme).

Another multi-enzymatic system was studied in an attempt to reach higher BMBA production rates. In the system proposed by

Turner *et al.* [72] (Figure 5 - 11), the transamination equilibrium between the amino-acceptor (here, BAP) and the amino-donor (D-Alanine) is also shifted by the consumption of the co-product. Pyruvic acid is reduced into lactic acid in a reaction catalysed by Lactate Dehydrogenase (LDH), in the presence of NADH cofactor. The latter is then regenerated by the glucose dehydrogenase (GDH) as in the previous experiments.

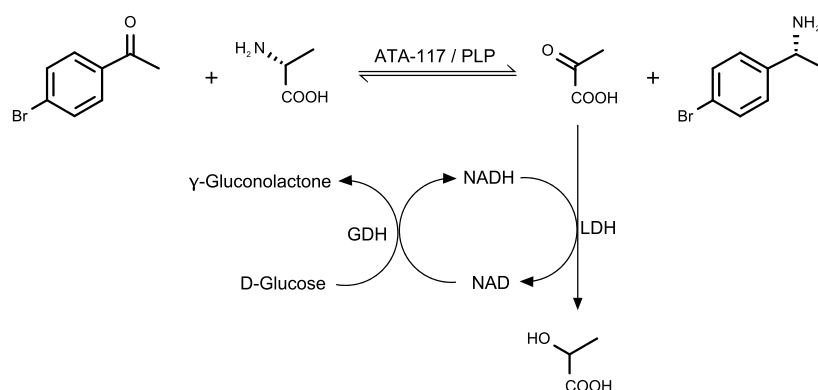


Figure 5 - 11 – Multi-enzymatic model (ATA/LDH/GDH). This reaction involves the BAP as amino-acceptor, and D-Alanine as amino-donor. The ATA-117 catalysed reaction allows the production of pyruvic acid. This co-product is then transformed into lactic acid by the LDH enzyme, using NADH as a cofactor. The NADH is recycled by the glucose oxidation reaction catalysed by the GDH enzyme.

Experiment was conducted using similar enzyme concentrations as Turner *et al.* [72]. R-BMBA production was quickly increased, reaching rapidly satisfying production levels (Figure 5 - 12). Indeed, using ATA-117, we reached 2.38 % yield in 10 minutes (corresponding to our standard contact time, 10 min, in flow mode). Catalytic efficiency at 10 minutes has also been calculated (using the relation detailed in the Introduction, Section 2.4.1) and reached $0.0118 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}_{\text{ATA117}}^{-1}$ and $0.0537 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}_{\text{HisTA}}^{-1}$ (batch duration was set to 10 min, for 0.11 mM BMBA and 5 mg ATA). We therefore decided to keep this system for an application in flow mode (*vide infra*).

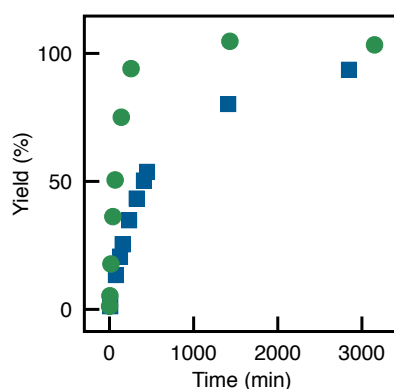


Figure 5 - 12 – Yield through the ATA – LDH – GDH system. (■) ATA-117; (●) His-TA.

2.3 Asymmetric synthesis in flow mode

Detailed reaction conditions are presented in Annexe 14.

Attempts for immobilizing the ATA-117/LDH/GDH system on monolith were conducted, and such biocatalyst was tested in flow mode, using our standard conditions (see Experimental, Section 2.3.2.2). The most promising APTES-functionalized monolith developed in Chapter II was used on each attempt of the present section (A250_D).

In a first set of experiments (Figure 5 - 13), after glutaraldehyde activation, the monolith was impregnated with a buffered solution containing 10 mg of each enzyme. Then, the monolith was washed with buffer. Finally, reaction medium for asymmetric synthesis was injected at 0.11 mL/min flow rate (BAP 5 mM, Alanine 50 mM, PLP 0.5 g/L, NADH 100 μ M, NAD 100 μ M, Glucose 50 mM).

Two kinds of transaminases were tested in combination with LDH and GDH: ATA-117, and a His-tagged ATA-117 (His-TA; see Chapter IV).

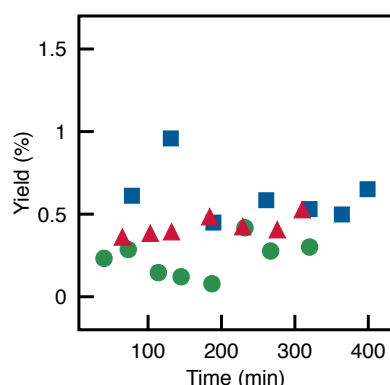


Figure 5 - 13 – Multi-enzymatic system (ATA/LDH/GDH) in flow mode. (■) Transaminase is ATA-117; (▲) Transaminase is His-TA; (●) Blank test (no enzyme).

In each case, the yield was very low (less than 1 %), but seemed to be slightly higher than the blank test, suggesting the occurrence of a slow biocatalytic activity.

Catalytic efficiency was calculated using the relation in the Introduction, Section 2.4.1: considering a product concentration of 0.05 mM (*i.e.* 1 % yield of 5 mM initial BAP concentration), a flow rate of 0.11 mL/min and a enzyme mass (ATA) of 10 mg (as the loading is not known as discussed in the Annexe 2), a catalytic efficiency of $0.00055 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}_{\text{ATA}}^{-1}$ is reached. This catalytic efficiency is 21 times lower than the batch efficiency (see Section 2.2). Thus we expected to improve it.

By considering the contact time used (10 minutes), we expected to reach 2.38 % yield in the ideal case (no enzyme deactivation). However, the yield (with enzymes; Figure 5 - 13) was observed around 1 % production, and it was hard to determined if this observed production was significantly over the blank (around 0.5 %). Then, if the obtained yield (with enzymes) was actually significantly higher than the blank production, the residual activity would be 42 %, which would be satisfying. Then, further experiments have been implemented in order to demonstrate that the conversion was significant (*vide infra*).

Biocatalyst (ATA-117/LDH/GDH on A250_D) was examined in order to determine if each enzyme is active on the monolith. For that purpose, specific reaction media were successively injected through the monolith (Figure 5 - 14). LDH and GDH enzymes reached high activity level (respectively 85 % NADH conversion and 100 % NADH

production), with a similar magnitude as the activity level reached when LDH and GDH are immobilized alone (Figure 5 - 14, right). On the contrary, ATA-117 was poorly active in the kinetic resolution way as BAP conversion was low (less than 2% BAP conversion was observed, to be compared with the 27.5 % obtained when ATA-117 was immobilized alone, TA-GA-A250_D-30d see Chapter II, Section 2.3). Therefore, it was suspected that other enzymes (LDH and/or GDH) could disturb the transaminase immobilization through competition at the monolith surface.

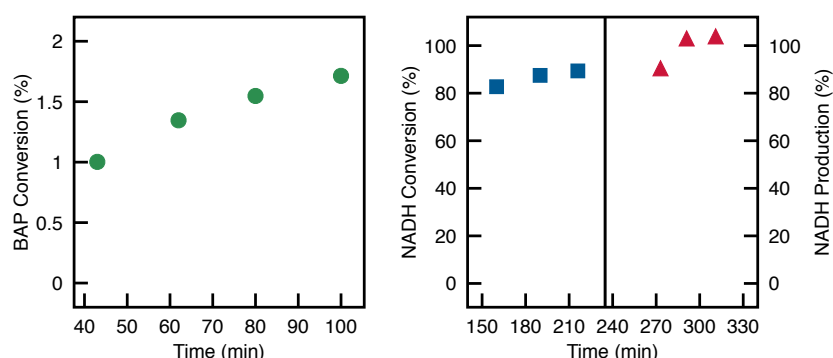


Figure 5 - 14 – Multi-enzymatic system (ATA/LDH/GDH) in flow mode. (●) Kinetic resolution on A250_D monolith impregnated by equimolar solutions of ATA-117, LDH and GDH. BAP yield is low. (■) NADH conversion by LDH, (▲) NADH production on the same monolith by GDH reached high activity.

Because competition between enzymes during the immobilization step was suspected, we conducted flow tests by changing the immobilization step (Figure 5 - 15): instead of using a buffered solution containing the three enzymes, we first impregnated the monolith with 50 mL of a single-enzyme solution (10 mg of ATA-117). Then, the monolith was impregnated with a solution containing the two other enzymes (10 mg each). However, R-BMBA production was still low. This result suggests that, if competition occurred between ATA and other enzymes, impregnating the transaminase before other enzymes does not allow avoiding it. The same experiment was repeated, changing the order of impregnation (LDH first or GDH first), but the activity in flow mode remained very low (Figure 5 - 15).

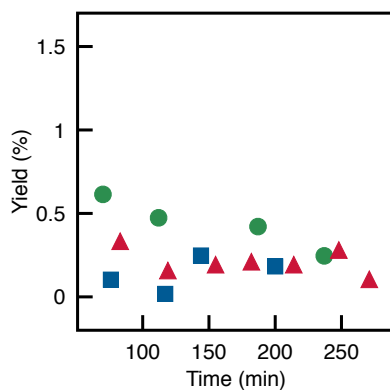


Figure 5 - 15 – Flow cascade using different impregnations sequences. (●) ATA-117 is impregnated first, then LDH and GDH; (■) LDH is impregnated first, then ATA-117 and GDH; (▲) GDH is impregnated first, then ATA-117 and LDH.

Low yield obtained on Figure 5 - 13 could be also explained by the low contact time used (leading to a low expected activity). Thus, contact time was increased by using 3 monoliths A250_D in series (Figure 5 - 16). Expected yield is 6.80 %. The activity was slightly higher than the conversion level obtained with only one monolith, thereby attesting that a biocatalytic transformation takes place in the system. However, the yield remained very low (less than 1 %).

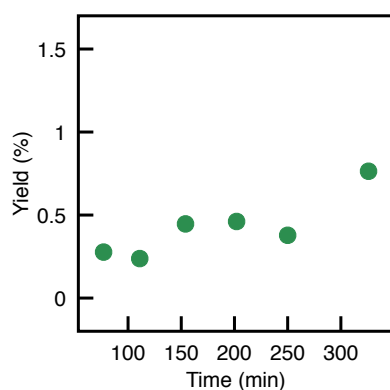


Figure 5 - 16 – Flow cascade using three A250_D monoliths in series.

From now on, it has been observed that LDH and GDH are effectively active on the monoliths, but they are not acting at the same concentration magnitude than transaminase (100 μ M NAD and NADH vs 5 mM BAP). Then, we hypothesized that LDH and GDH were not able to displace the chemical equilibrium in those

conditions. Accordingly, we implemented an experiment where ATA-117 is the only enzyme that is immobilized on the monoliths (Figure 5 - 17). The two other enzymes, LDH and GDH, are fed in the reaction medium with all the reactants, in high concentration (see Annexe 14). Then, we expected to bring enough LDH and GDH to drive the equilibrium (enzyme concentrations are the same used for batch reaction, see Figure 5 - 12). However, once again, BMBA production remains very weak. It was suspected that LDH and GDH were immobilized in too low amount to efficiently displace the equilibrium towards the asymmetric synthesis. Obviously, continuously feeding the enzymes in high concentration is not viable from an industrial point of view. However, it allowed us to perform the asymmetric synthesis by hook or by crook. As a perspective, one would dwell on LDH and GDH immobilization optimization.

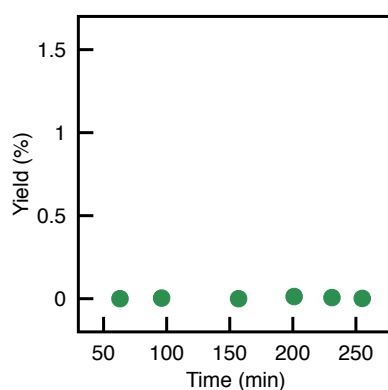


Figure 5 - 17 – Multi-enzymatic system (ATA/LDH/GDH) in flow mode. LDH and GDH enzymes are fed in the reaction medium. ATA-117 is immobilized.

A similar experiment was conducted, but using 2 monoliths in series and by dividing the rate flow by two. So, the contact time is 40 minutes, and expected yield is 8.90 % (Figure 5 - 18). A slight increase in activity was observed (yield reached 1.2 %).

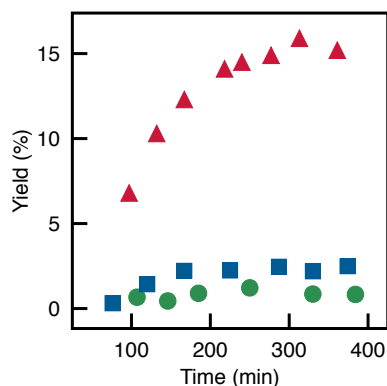


Figure 5 - 18 – Multi-enzymatic system (ATA/LDH/GDH) in flow mode. LDH and GDH enzymes are fed in the reaction medium while transaminase is immobilized. Two monoliths are implemented in series and rate flow is 0.055 mL/min. Various transaminases are tested : (●) ATA-117, (▲) His-TA, and (■) HEwT.

Attempts to further increase this BMBA production were conducted, using other transaminases, respectively His-TA and HEwT (see Introduction Section 0 and Chapter IV). Using HEwT only 2.5 % yield was reached. However, significant yield was obtained with His-TA (up to 16 %). Corresponding catalytic efficiency was $0.0044 \mu\text{mol}_{\text{BMBA}} \cdot \text{min}^{-1} \cdot \text{mg}_{\text{TA}}^{-1}$ (if assuming the mass of TA enzyme is set to 10 mg). Long stabilization time was required (240 minutes), due to the higher contact time (reactant takes up to 4 hours to reach the end of the flow setup). After the stabilization time, production seemed to be stable over time. Those results suggest that, using a highly pure transaminase (see Chapter IV), more efficient immobilization is obtained leading to higher transamination efficiency.

3 Conclusion

In this chapter, we first had to select a system to drive the equilibrium towards the asymmetric production of chiral amines. Several systems have been studied in batch mode.

Mono-enzymatic systems were not convenient: they did not lead to sufficient equilibrium displacement and required the use of other catalysts.

Multi-enzymatic systems were then studied. After various optimizations (on enzymes and substrates concentrations), the TA-YADH-GDH was quite satisfactory (good yield was obtained). Finally, the system TA-LDH-GDH allowed reaching relatively high reaction

rates and high conversion levels in the batch mode. It was therefore selected for further flow experiments.

Flow transamination in the asymmetric synthesis way was conducted using TA, LDH, and GDH enzymes co-immobilized on the same monolith. However, BMBA production was very low, only slightly higher than the blank experiment run in the absence of enzymes. This was true; either when all three enzymes were co-immobilized simultaneously, and when immobilized in two steps, whatever the order of enzyme immobilization. Contact time was increased in order to enhance the activity, resulting only in a marginal increase in activity.

In a different approach – stimulated by a hypothesis that not enough enzymes were immobilized on the surface – the enzymes used to shift the thermodynamic equilibrium (LDH and GDH) were injected in relatively high concentrations in the reaction medium instead of being co-immobilized. Three transaminases were tested (ATA-117, His-TA and HEwT). While ATA-117 transaminase still showed low BMBA production, HEwT and His-TA transaminases allowed to reach satisfying activities by this method (respectively 2.5 % and 16 %). This constitutes the first example of flow synthesis of chiral amine for such system where the thermodynamic equilibrium is so unfavorable.

Chapter VI – Production and immobilization of TAL enzyme for flow kinetic resolution of chiral amines

1 Introduction

The last objective of this PhD Thesis was to transfer our technology to another biocatalytic system (see Introduction, Section 3). In that purpose, we wanted to develop a heterogeneous biocatalyst (suitable for flow transformations) for the production of organic compounds of industrial interest. Taking advantages of lessons learnt in the Chapter IV, we had to develop such biocatalyst, starting from a pure enzyme extract. Thus, we have produced the enzyme ourselves (in the IPB laboratory, Halle, Germany).

Tyrosine ammonia-lyase (TAL) was considered as the ideal candidate for the development of new heterogeneous flow biocatalyst. TAL (EC. 4.3.1.23/25 [38]) catalyses the formation of α,β -unsaturated acids from amino acids (releasing ammonia) [213,214]; or the reverse reaction, *i.e.* the asymmetric addition of ammonia to arylacrylates (*e.g.* coumaric acid), forming amino acid of high value (L-tyrosine) [38] (Figure 6 - 1).

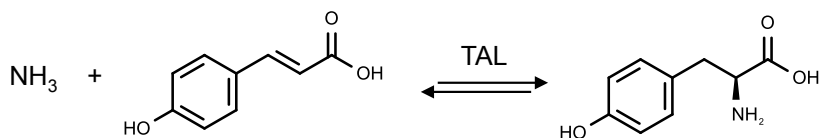


Figure 6 - 1 – Reaction catalysed by the TAL. The asymmetric amination of coumaric acid, leading to the formation of L-tyrosine.

TAL enzymes are part of the ammonia-lyase category, that contains also Phenylalanine Ammonia-lyases (PAL) and Histidine Ammonia-lyases (HAL) [215]. Those enzymes follow the kinetic of Michaelis-Menten [216]. While PAL enzymes have large substrate specificity, TAL and HAL have a very narrow specificity (actually, limited to tyrosine and histidine, respectively) [214]. In living organisms, TAL are involved in several metabolic pathways such as caffeic acid biosynthesis.

Thermodynamically, the deamination of tyrosine is favoured [217], thus leading to the production of coumaric acid. Coumaric acid is of relevant industrial interest. Indeed, it is involved in the synthesis of many aromatic compounds such as stilbenes (*e.g.* resveratrol),

flavonoids (e.g. naringenin) and plastic precursors (e.g. p-hydroxystyrene) [213]. Coumaric acid is also a precursor of antibiotics in microorganisms and protein cofactor [215].

Considering the implementation of strategies for the displacement of chemical equilibrium towards the asymmetric synthesis of L-tyrosine, the use of TAL for the production of tyrosine (starting from coumaric acid and ammonia) is also of great industrial interest. Indeed, L-tyrosine is a recurring pattern in numerous bioactive drugs [38,217] such as anti-diabetes, antiviral hepatitis C and anticoagulant precursors.

Like most enzymes, TAL enzymes are highly enantioselective and work in mild conditions: aqueous media, low temperature (30 °C) neutral pH (7.5). However, TAL suffered from a too weak enzymatic activity (reaction rates) for an efficient industrial application. Recently, Jendresen *et al.* [213] identified 22 new TAL sequences. After insertion of the sequences in bacteria and yeasts, some TAL produced exhibited a high activity (allowing to consider a further industrial use of TAL). Among those enzymes, the TAL from the microorganism *Flavobacterium johnsoniae* (FjTAL) has particularly demonstrated a high efficiency. Also this new TAL shows a larger selectivity (using tyrosine and tyrosine esters as substrates) than other TAL.

The TAL mechanism has been elucidated: three amino acids residues are essential for the catalytic act: one serine and two tyrosine residues [218]. TAL does not use an external cofactor (unlike transaminases that use PLP). However, a prosthetic group (3,5-dihydro-5-methylidene-4H-imidazole-4-one, "MIO") is formed within the enzyme structure (three amino acids -alanine, serine and glycine- are folded, and the MIO group is formed after two dehydration steps) [218]. The main advantage of this prosthetic group is that it is permanently linked to the enzyme and does not need to be fed continuously (if considering a flow device).

In this chapter, we have first produced the efficient FjTAL (afterwards, we simply call it "TAL"). The gene used (graciously given from University of Denmark) contained a His-tag for purification. Thus we aimed at producing a highly pure enzyme.

TAL enzymes are homo-tetrameric enzymes. Each enzyme bears four active sites, located at the interface of the monomers (the active site of transaminases –that are dimeric enzymes- is also located at

the interface between monomers, see Introduction, Section 2.2.2) [217].

Yet, the structure of this TAL is unknown. We used a homology model for the surface composition prediction. Using BLAST tool [219], we found a homologue TAL from *Streptomyces globisporus* (SgTAL, 36 % identity). The PISA tool [60] showed the surface composition regarding the SgTAL structure. Three lysine residues are exposed to the surface (as for transaminases). Thus, covalent immobilization using APTES and glutaraldehyde can be considered.

Thus, the immobilization of TAL can be considered in our supports (developed in our lab, *i.e.* Si(HIPE) monoliths grafted with amino moieties (A250_D)).

Finally, immobilized TAL is tested under flow conditions, using the tyrosine deamination as model reaction.

2 Results and discussion

2.1 TAL production and purification

Three series of TAL expressions have been achieved and named respectively A, B and C. After purification, enzyme solutions were divided into aliquots labeled « TAL aliquots » from 1 to 11 (see Table 7). Protein concentration was determined by BCA Assay.

Table 7 – Expression batches, TAL aliquots correspondence and TAL concentrations in aliquots.

Expression batches	TAL aliquots	TAL concentration (g/L)
A	1	21.9
	2	26.4
	3	22.8
B	4	5.3
	5	5.9
	6	6.0
	7	7.8
C	8	6.3
	9	6.0
	10	5.9
	11	5.8

It resulted that the first expression batch seemed to lead to higher protein expression as final TAL aliquots contain around 4 times more protein than batches B and C. However, an unexpected precipitation was observed during the purification step of expression batch A. Indeed, it seemed that the wrong buffer have been used for one step of purification, with a too low salt concentration, that led to protein precipitation (that would be then irreversibly denatured).

Protein purification efficiency was evaluated through SDS Gels separation (Figure 6 - 2).

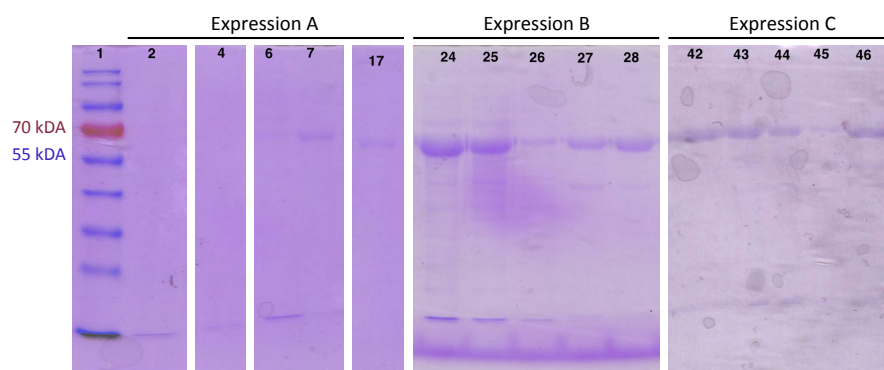


Figure 6 - 2 – SDS Gels. PageRuler protein ladder is in well 1. Purification aliquots from expression batch A are: 2 (Flow-through, FT), 4 (Washing flow, W), 6 (Lysate, L), 7 (Elution Flow, E) and 17 (purified TAL2). Purification aliquots from expression B are: 24 (L), 25 (FT), 26 (W), 27 (E) and 28 (TAL4). Purification aliquots from expression C are: 42 (L), 43 (FT), 44 (W), 45 (E) and 46 (TAL11). See Experimental, Section 2.1.1.2 for detailed protocols.

As TAL has a molecular weight of 60 kDa, TAL band should appear between 70 and 55 kDa markers (indicated by PageRuler).

For expression batch A, TAL band is clearly observed in L, E and TAL2 wells. No other bands are clearly observed in those wells. This suggests that the final TAL2 aliquot is pure enough. No other bands are observed in the other wells. This could be due to a too low loading in the wells. Thus, this suggests that the TAL has been successfully purified as all the protein has been collected into the aliquots E (before enzyme re-concentration, see Experimental, Section 2.1.1.2) and TAL2 (after re-concentration, aliquot ready to use for catalysis). No band is observed in other aliquots, thus no enzyme was lost during the purification (or at least, not in a detectable concentration). Also, as only one band is observed for aliquots E and TAL2, this suggests that the enzyme is quite pure (or at least, no other protein is co-purified in those aliquots).

For expression batch B, TAL bands are observed between 70 and 55 kDa in each wells. Large TAL bands are observed in L and FT wells indicating that this expression led to a high TAL production. However, due to the limited binding capacity in the cobalt column, a large amount of TAL is lost during the purification. TAL enzyme is also observed in W well, confirming the suspected saturation of the purification column. Moreover, slight protein band is observed around 40 kDa for E and TAL4 wells. This indicated that the final TAL aliquot is not totally pure. This less good purification could be due to the

saturation of the purification column. The same is observed for expression batch C.

Thus, the batch A was successfully purified and exhibited a better purity than batches B and C. However, as the aliquots from batch A were precipitated (TAL1, TAL2 and TAL3), we suspected a loss in activity for those aliquots.

2.2 Activity assessment

Three TAL-catalysed deamination reactions were investigated. As substrates, we used L-Tyrosine (TYR), L-Tyrosine methyl ester (Me-TYR) and L-Tyrosine ethyl ester (Et-TYR).

Products absorb in UV range and can then be detected by spectrophotometry. Respectively, products and their maximum of absorbance are coumaric acid (Coum; 290 nm), coumaric methyl ester (Me-Coum; 310 nm) and coumaric ethyl ester (Et-Coum; 310 nm). TAL aliquots activity is assessed by measuring product concentration over time and calculating the initial reaction rates (Table 8).

Table 8 – Rates and specific activities of TYR, Me-TYR and Et-TYR deaminations catalysed by TAL.

TAL aliquots	Concentration (g/L)	Initial rate ($\mu\text{M}_{\text{PRODUCT}} \cdot \text{min}^{-1}$)			Specific initial activity ($\mu\text{mol}_{\text{PRODUCT}} \cdot \text{min}^{-1} \cdot \mu\text{mol}_{\text{TAL}}^{-1}$)		
		Coum	Me-Coum	Et-Coum	Coum	Me-Coum	Et-Coum
1	0.1	0.12	0.05	0.09	0.07	0.03	0.05
2	0.1	0.09	0.04	0.07	0.06	0.03	0.04
3	0.1	0.11	0.07	0.08	0.07	0.04	0.05
4	0.1	0.95	0.63	0.64	0.57	0.38	0.39
5	0.1	1.00	0.67	0.66	0.60	0.40	0.40
6	0.1	0.84	0.64	0.64	0.50	0.38	0.39
7	0.1	0.76	0.58	0.55	0.46	0.35	0.33
8	0.1	0.92	0.73	0.53	0.55	0.44	0.32
9	0.1	0.72	0.57	0.45	0.43	0.35	0.27
10	0.1	0.78	0.59	0.51	0.47	0.36	0.31

11	0.1	0.69	0.57	0.44	0.42	0.34	0.26
No TAL (blank)	0	0.00	0.00	0.01	/	/	/

As expected, TAL1, TAL2 and TA3 aliquots exhibit a relatively low specific activities for each substrate (around 0.07, 0.03 and 0.05 $\mu\text{mol}_{\text{Product}} \cdot \text{min}^{-1} \cdot \mu\text{mol}^{-1}_{\text{TAL}}$ for TYR, Me-TYR and Et-TYR respectively). This is not surprising as protein precipitation was observed during the purification steps of these batches (the buffer used for desalting column equilibration was not salty enough, this was corrected for further purifications).

Other TAL aliquots show higher activities (0.50, 0.38 and 0.33 $\mu\text{mol}_{\text{Product}} \cdot \text{min}^{-1} \cdot \mu\text{mol}^{-1}_{\text{TAL}}$ on average for TYR, Me-TYR and Et-TYR respectively). Higher activity is observed towards TYR. No significant difference is observed towards Me-TYR and Et-TYR. Aliquots from batch B are slightly more active than aliquots from batch C.

The ratio $k_{\text{CAT}}/K_{\text{M}}$ is evaluated in order to assess catalytic efficiency (Table 9).

Table 9 – Mean catalytic efficiencies depending on substrates, and k_{CAT} and K_{M} constants determination.

Substrate	Mean* k_{OBS} (min^{-1})	C_{TAL} (M)	$k_{\text{OBS}}/C_{\text{TAL}} =$ $k_{\text{CAT}}/K_{\text{M}}$ ($\text{min}^{-1} \cdot \text{M}^{-1}$)	K_{M} (μM)	k_{CAT} (min^{-1})
TYR	0.032	1.66E-06	19161.8	36.7	0.70
Me-TYR	0.013	1.66E-06	8139.3	59.1	0.48
Et-TYR	0.011	1.66E-06	6676.6	68.7	0.46

*Excluding data from expression batch A.

The Michaelis-Menten constant (K_{M}) was determined by measuring deamination rates while varying substrate concentration (Figure 6 - 3). Aliquot TAL4 was used for this determination. Non-linear regression was used for K_{M} determination.

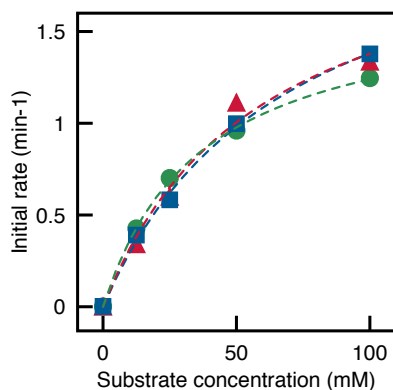


Figure 6 - 3 – Initial rate depending on substrate concentration for the three substrates. TYR (●), Me-TYR (■) Et-TYR(▲).

Obtained K_M values are in the typical range of K_M for TAL enzymes (ranging from 16 to 100 μM) [220]. However, catalytic efficiency values are much lower than expected (around 10^4 vs $6 \cdot 10^5$ $\text{min}^{-1} \cdot \text{M}^{-1}$) [220].

2.3 Immobilization and flow deamination

The results presented in this section have been conducted using TAL aliquot from expression batch B (TAL aliquots 4 to 7). The biocatalysts were tested using the tyrosine deamination as model reaction (producing coumaric acid and ammonia).

TAL have been first immobilized through simple adsorption on Si(HIPE) monolith. Flow experiments have been conducted using various TAL masses in the enzyme impregnation solution (Figure 6 - 4, see Experimental, Section 2.3.2.2).

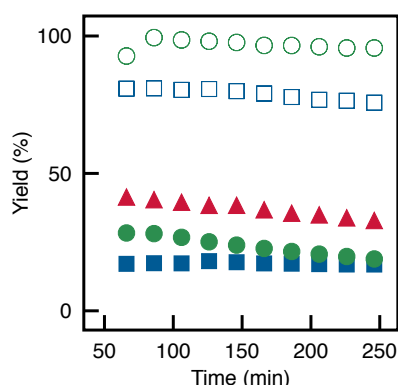


Figure 6 - 4 – The yield of tyrosine deamination over time, depending of the TAL mass injected in the impregnation solution. TAL were immobilized on Si(HIPE) monoliths through simple adsorption. TAL masses are 0 (■), 0.185 (●), 0.375 (▲), 0.75 (□) and 1.5 mg (○).

The blank (no enzyme in the impregnation solution) led to a slight conversion (due to the spontaneous tyrosine deamination). The yields were quite stable over time for each TAL mass impregnated. The relation between the mass used for enzyme impregnation and the yield shows that the yield increases with the enzyme mass involved in the impregnation solution (Figure 6 - 5). The yield seems to stabilize (around 100 %) when the enzyme mass exceeds 1.5 mg. This indicates that for 1.5 mg, the maximal yield is obtained, and more enzyme does not allow to increase the conversion rate.

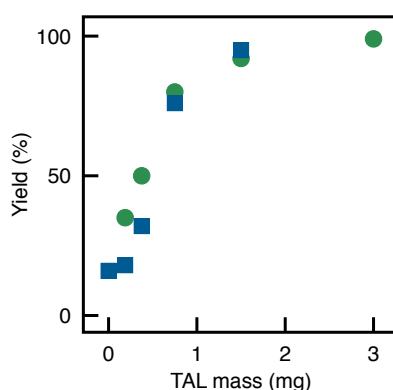


Figure 6 - 5 – The yield of tyrosine deamination catalysed by immobilized TAL (eventually at the stabilization), depending on TAL mass involved in the impregnation solution. Supports are Si(HIPE) (■) or A250D (●).

TAL was also immobilized on A250_D supports (developed in the Chapter II), in combination with glutaraldehyde pre-activation (Figure 6 - 6). For high TAL masses in the impregnation solution, a relatively long stabilization time is observed, during which the yield progressively increases before reaching a stable value. This could be assigned to a slight interaction between reactants or product with the monolith surface (grafted with APTES and activated with glutaraldehyde), e.g. electrostatic interactions between tyrosine (negatively charged) and amino moieties (positively charged at pH 7.5) that have not reacted with glutaraldehyde. Regarding the yield at the stabilization plateau *versus* the mass injected in the impregnation solution (Figure 6 - 5), the yield increases with the enzyme mass up to 1.5 mg TAL, before reaching a plateau at its maximal conversion level (100 % yield). This trend is similar to the one observed with non-functionalized monolith (Si(HIPE)).

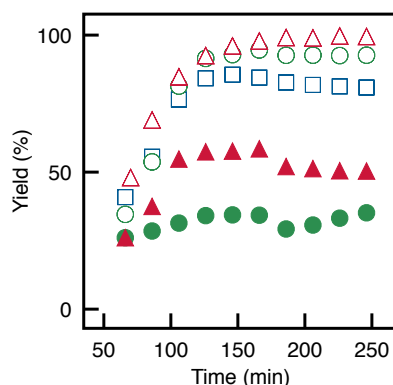


Figure 6 - 6 – The yield of tyrosine deamination over time, depending of the TAL mass injected in the impregnation solution. TAL were immobilized on Si(HIPE) monoliths functionalized with APTES (A250_D) and pre-activated with glutaraldehyde (covalent grafting). TAL masses are 0.185 (●), 0.375 (▲), 0.75 (□), 1.5 mg (○) and 3 mg (△).

TAL loadings have been measured for each flow experiment (Figure 6 - 7). For both catalysts (TAL-Si(HIPE) and TAL-A250_D-GA), a similar trend is observed : the yield increases linearly with the TAL loading in the monolith, up to the maximal conversion rate (100 % yield). A further increase in the loading (over 1.5 mg) did not allow increasing the yield as it has already reached its maximum. We suspect that the catalyst is saturated in enzyme when using more than 1.5 mg enzyme (both for Si(HIPE) and A250_D-GA carriers).

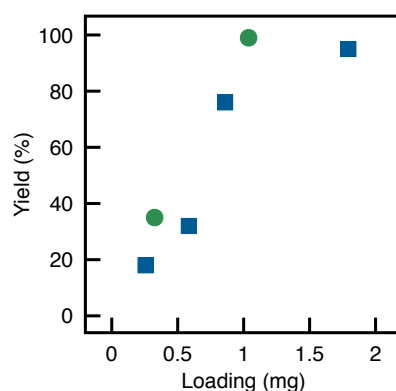


Figure 6 - 7 – Tyrosine deamination yields as function of TAL loadings in the monoliths. Supports are Si(HIPE) (■) or A250D (●).

A last experiment was conducted in order to assess the stability of the TAL-biocatalysts (Figure 6 - 8). The flow tyrosine deamination was first conducted for 8 hours. Then, the flow rate was stopped for 12 hours (the biocatalysts were still placed at 30 °C in the water bath). After this interrupted time, the flow rate was re-set at 0.11 mL/min (as before) and the yield was measured over time for 4 additional hours. The biocatalysts were both loaded with 3 mg TAL in the impregnation solution.

For TAL-Si(HIPE) and TAL-GA-A250D, the first yields (before interruption) were respectively around 80 % and 90 %. That was a bit lower than the yield expected (the maximal yield). This could be due to a slight variability in the biocatalyst preparation. For the TAL-Si(HIPE) catalyst, the yield after interruption was lower than the initial yield, and decreasing over time (from 70 to 60 %). For the covalently immobilized TAL (TAL-A250D-GA), the yield observed after interruption was still high and relatively stable (around 80 %). This is assigned to the efficiency of the covalent immobilization method: after long period of time, enzymes are not leached because the covalent bond is more efficient. Concerning the simple adsorption, the weak bond between enzymes and surface would not have allowed to efficiently retain the enzymes after a long period: enzymes might have unhooked in the solution during the interruption. Thus, when the flow rate was re-set after interruption, enzyme may have been leached in the flow rate over time, leading to a lower, and decreasing, yield over time.

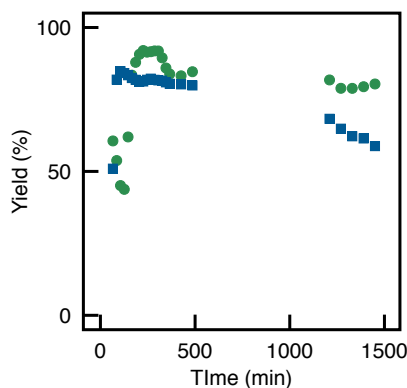


Figure 6 - 8 – Yield of tyrosine deamination over time, and after a long period of interruption (no flow rate). Supports are Si(HIPE) (■) or A250D (●).

3 Conclusion

In conclusion, the use of tyrosine ammonia-lyase for the production of coumaric acid is on increasing interest due to its potential use in organic synthesis. The use of TAL in the non thermodynamically pathways (*i.e.* the enantioselective production of tyrosine) is a challenge for the pharmaceutical industry (need for strategies for equilibrium displacement).

As for the transaminases, TAL are involved in the chiral amine synthesis (but starting from arylacrylates, instead of ketones or aldehydes).

FjTAL was produced through bacterial transformation, protein expression and purification (using His-tag). Activity has been assessed successfully in batch mode.

TAL have been immobilized on carrier through either simple adsorption or covalent immobilization. Then, the biocatalysts were assessed in flow mode, using the same device used for flow transaminations. Indeed, the objective was to demonstrate the applicability of the developed TA flow device, with minimal tailoring for applicability to TAL enzymes.

Surprisingly, the simple adsorption led to active flow biocatalyst (whereas transaminases were totally inactivated following simple adsorption). Thus, it is assumed that the TAL immobilized exhibit a strong stability towards laboratory manipulation, that is a required quality for industrial application. TAL were also active when

covalently immobilized (through APTES/glutaraldehyde). The biocatalysts were highly active (as the maximal yield was reached), and were stable after long period of interruption. These efficient biocatalysts demonstrated the facile applicability of TA-biocatalysts developed for flow transamination reactions.

Conclusions

The general aim of this PhD thesis was the development of efficient heterogeneous biocatalysts for the greener production of industrially relevant chiral amines in continuous flow mode. In particular, this work tackles several challenges: controlling the immobilization of transaminases; performing transaminations in flow mode; and displacing the chemical equilibrium towards the production of chiral amine of industrial interest (asymmetric synthesis).

Firstly, we have developed a method to perform flow enzymatic reactions. The choice of Si(HIPE) materials and their shape and size was suitable for enzymatic flow reactions at the laboratory scale: our flow device was easy to set-up, allowed simultaneous parallel reactions (up to three). The flow was verified to follow an ideal plug-flow profile.

Several immobilization methods have been studied on transaminases (in flow mode). First, the simple adsorption of transaminases (ATA-117) led to poor enzyme activity. For a better control on immobilization, the chemistry of the monolith surface needed to be modified. The functionalization process allowed the grafting of various chemical functions at the silica surface (e.g. amines, epoxides and methyl groups). Initially the functionalization process followed the method of Ungureanu *et al.*, that globally consisted in dipping the monoliths in the grafting solution (under vacuum) and to let them impregnate in the solution for one day. Monoliths functionalized by this way allowed the enzyme immobilization. However, a poor dispersion of organosilane (APTES) was observed both macroscopically and by several characterization techniques. An optimized method was developed; which consisted in the rapid dipping of monoliths into the solution until they were soaked. They were then removed and let for ageing under controlled atmosphere. Moreover, the water content (known to influence the silanization reaction) was controlled by using dry monoliths and water saturated solvent. This functionalization method (Method D) allowed to obtain and improved organosilane dispersion (observed macroscopically and by characterizations) and good repeatability (assigned to the better control on organosilane and water inputs).

Attempt to immobilize transaminases through electrostatic interactions was then conducted (using monoliths functionalized with APTES, that is positively charged under pH 8, while transaminases are negatively charged at the same pH. The biocatalysts were active.

Conclusions

In order to get more efficient biocatalysts, covalent grafting was attempted, and has been studied on enzymes in two different ways: the use of APTES-grafted monoliths (amino moieties) in combination with glutaraldehyde, forming imines bonds (APTES/GA); and the use of GLYMO-grafted monoliths (epoxide rings). GLYMO-grafted monoliths gave unsatisfying results (poor activity). We suspected that this method deactivated the enzymes (for an unknown reason). APTES/GA monoliths gave the best biocatalytic performance. The method of APTES functionalization, and the associated control on the dispersion of amino group at the surface, was shown to have a crucial impact on the final biocatalytic performance. The best carrier was A250_D in combination with glutaraldehyde. The link between the carriers (thus exhibiting an aldehyde moiety after APTES pre-activation with glutaraldehyde) and the enzyme is strong enough to anchor the enzymes. As no deactivation was observed over time, we suspect that many links between carrier and enzymes are involved in the immobilization of each enzyme. Moreover, the glutaraldehyde acts as a spacer, a long molecule that allows the enzyme to stay far from the solid carrier (and thus, could allow some spatial movements that may be crucial for enzymatic activity). Also, glutaraldehyde, as a spacer, allows avoiding the enzyme to be in direct contact with the carrier (that may be unfavourable for enzyme conformation, as assumed for simple adsorption method). This method was successfully applied to the immobilization of transaminases and tyrosine ammonia-lyase (TAL), both containing lysine residues at their surface. Thus, we assumed that this immobilization method could be applied to any enzyme that exhibits such amino functions.

Hydrophobic monoliths have been synthesized by two methods (one-pot method and post-grafting method). Only the post-grafting method led to workable monoliths. The post-grafting of MTES (methyls functions) allowed effectively synthesizing more hydrophobic materials. However, they show no advantage for enzyme immobilization (when combined to APTES-grafting): the presence of MTES did not change the efficiency of APTES-biocatalysts. When MTES was employed alone on the silica surface, no activity was detected. It was assumed that hydrophobicity of the surface did not affect the activity of ATA-117 (although it contains many hydrophobic residues at its surface). This could be due to the fact that, thanks to the glutaraldehyde spacer, the enzyme is not affected by the composition of the surface.

The use of purified enzymes (His-TA and TAL) allowed obtaining high yields (maximal yields were reached). Indeed, more enzymes could be immobilized per gram of extract. Also, pure enzyme stock

avoids contamination by undesirable compounds (and thus, potential interferences during different chemical steps, *i.e.* enzyme immobilization and enzymatic reactions).

One main limitation of this work is the difficulty to evaluate the enzyme loading (*i.e.* the amount of enzyme effectively immobilized into the monolith). We assumed that this issue was due to an aggregate dissociation when passing through the flow device, in combination with inappropriate quantification methods (that are not sensitive enough for low enzyme concentration). Improvements on the loading evaluation are welcome, and should allow determining catalytic efficiency with better accuracy.

Attempts for performing the asymmetric synthesis of chiral amines were conducted. An efficient strategy using LDH and GDH as proposed in the Literature was selected and demonstrated again in batch mode for our model reaction. The asymmetric synthesis of R-BMBA was successfully conducted when using the home-produced His-TA enzyme. In our attempt to transfer the method in flow mode, we could show that the three enzymes could be immobilized individually or together and maintain activity. Yet, when the three enzymes (His-TA, LDH, GDH) were co-immobilized, only a very low activity towards the chiral amine was detected. We suspect that LDH and GDH need to be immobilized in larger amount (as compared with TA) to promote the cascade reaction and shift the thermodynamic equilibrium. Nevertheless, using high concentrations of lactate dehydrogenase (LDH) and glucose dehydrogenase (GDH) in the reaction medium, we were able to demonstrate significant yields of production of the chiral amine in the flow mode. While this is a first example of flow mode synthesis of a thermodynamically unfavourable chiral amine, it is clear that the system is not yet optimized, since two of the three enzymes are free in the reaction media, and therefore can not be maintained in the fixed bed reactor and used for prolonged times.

Finally, we successfully performed the transfer of our technology (enzyme immobilization on macrocellular silica monolith and performing enzymatic reactions in flow mode) to the synthesis of other organic compounds of industrial interest (coumaric acid) using another enzyme (TAL). Immobilized TAL were highly active and stable over time (using A250D/glutaraldehyde carrier). In consequence, we propose that our method could be applied to other enzymatic transformations in flow mode. The main conditions are to use pure enough enzyme extract, and to choose an immobilization method that takes the enzyme surface composition into account.

Perspectives

A general method for enzyme immobilization (covalent grafting) and the achievement of flow enzymatic reactions has been developed in this work. Yet, some improvements could be provided, at different levels, in order to get a more efficient biocatalytic flow system.

Concerning the silica functionalization, a method combining the Methods C and D could be considered: by taking advantage from Method C, silica with high silanol content is obtained. By using such rehydroxylated silica, but grafted with the Method D (control on water inputs during the APTES-functionalization), one expects to get monoliths with higher APTES content (leading to higher enzyme loading) while keeping a satisfying repeatability (as obtained using the Method D). Also, the optimal water inputs during the functionalization step should be determined (e.g. by working under controlled atmosphere to saturate toluene at different water contents).

Regarding to the production of hydrophobic monoliths, we suggest assessing biocatalytic efficiency without glutaraldehyde (GA). Indeed, GA acts as a spacer and could keep the enzyme far from the carrier surface, thus the enzyme may not be affected by the change in surface polarity at this distance. Also, we suggest the use of lower MTES concentration for the one-pot synthesis. Indeed, high MTES concentration led to brittle monoliths, while a potential effect on enzyme immobilized is suspected even at low methyl concentration. MTES could also be inserted in the organic phase (dodecane) while forming the emulsion, thus allowing a better dispersion of the methyl groups at the silica surface (without affecting the aqueous/TEOS phase composition). Finally, one could use other hydrophobic moieties, such as octyl moieties to confer hydrophobicity to the carrier.

Enzyme immobilization step can be improved for getting even better enzymatic performance. First, the hypothesis of cold deactivation must be elucidated: by performing a size-exclusion chromatography at 4 °C and 30 °C, one could know if the enzyme dimer is indeed being dissociated at low temperature. Then, if a so-called cold denaturation occurs, the enzyme immobilization must be conducted at high temperature (and we suggest to find the optimal impregnation temperature). Alternatively, the gain in biocatalytic performance obtained when immobilizing the enzymes at high temperature could be assigned to the kinetic-dependent immobilization (reaction between GA and enzyme). Thus, a kinetic

study must be conducted to identify the optimal temperature for covalent grafting, or one must extend the enzyme impregnation duration to allow sufficient amount of enzyme to be immobilized. In any cases, glutaraldehyde concentration and flow rate optimization should be performed. Concerning the His-tag immobilization, it is suggested to increase the metal concentration on the monoliths (e.g. by optimizing the Lys-NTA grafting).

Also, the issue of enzyme loading measurement must be resolved. Indeed, it is important to know about the enzyme loading for comparing biocatalysts efficiencies. UV measurement usually give accurate concentration values. However, the cofactor, PLP, also absorbs in UV range. One option could be to derivatize the protein for shifting the UV absorbance range out of the PLP absorbance range or the use a fluorescent tag.

Concerning the industrial viability of the whole process, one must optimize the co-immobilization of several enzymes (TA, LDH and GDH) on the same support. The enzymes concentrations should be adjusted, by taking account of the ability of each enzyme to be immobilized on the surface. To that end, a deep study on the immobilization of each enzyme on the silica monolith should be conducted, to identify the best method (or at least, a common acceptable working window). Also, the expensive cofactor PLP (that was fed continuously in our work) should be either recovered and recycled after the reaction, or co-immobilized on the enzyme carrier.

References

- [1] L. van den Biggelaar, P. Soumillion, D.P. Debecker, *Catalysts* 7 (2017) 54.
- [2] T.C. Nugent, *Chiral Amine Synthesis: Methods, Developments and Applications*, Wiley-VCH, Mörlenbach, 2010.
- [3] Sanofi-Aventis, Sanofi-Aventis Confirms Qual. Supply Plavix®, Iscover® Clopidogrel Winthrop® (2010).
- [4] J.M. Grohol, *Top 25 Psychiatr. Medicat. Prescr.* 2011 (2011).
- [5] P.T. Anastas, J.C. Warner, *Green Chemistry: Theory and Practice*, Oxford University Press, New York, 1998.
- [6] Iwao Ojima, *Catalytic Asymmetric Synthesis*, John Wiley & Sons, Hoboken, 2010.
- [7] C.K. Savile, J.M. Janey, W.R. Jarvis, J.C. Colbeck, A. Krebber, F.J. Fleitz, J. Brands, *Science* 329 (2010) 305–310.
- [8] I. Slabu, J.L. Galman, R.C. Lloyd, N.J. Turner, *ACS Catal.* 7 (2017) 8263–8284.
- [9] T. Sehl, H.C. Hailes, J.M. Ward, R. Wardenga, E. Von Lieres, H. Offermann, R. Westphal, M. Pohl, D. Rother, *Angew. Chemie - Int. Ed.* 52 (2013) 6772–6775.
- [10] L.A. Nguyen, H. He, C. Pham-Huy, *Int. J. Biomed. Sci.* 2 (2006) 85–100.
- [11] I. Slabu, J.L. Galman, C. Iglesias, N.J. Weise, R.C. Lloyd, N.J. Turner, *Catal. Today* 306 (2018) 96–101.
- [12] D.L. Nelson, M.M. Cox, *Lehninger Principles of Biochemistry*, Macmillan Learning, New-York, 2013.
- [13] Roger Crossley, 48 (1992) 8155–8178.
- [14] J.S. Lee, W.K. Yang, E.Y. Han, S.Y. Lee, Y.H. Park, M.A. Lim, H.S. Chung, J.H. Park, *Forensic Sci. Int.* 173 (2007) 68–72.
- [15] W.F. Kean, H.E. Howard-Lock, C.J.L. Lock, *Lancet* 338 (1991)

References

1565–1568.

- [16] M.J. Cooper, M W Anders, *Life Sci.* 15 (1974) 1665–1672.
- [17] W.H. De Camp, *J. Pharm. Biomed. Anal.* 11 (1993) 1167–1172.
- [18] U.S. Food and Drug Administration, *Chirality* 4 (1992) 338–340.
- [19] H.U. Blaser, F. Spindler, M. Studer, *Appl. Catal. A Gen.* 221 (2001) 119–143.
- [20] H.U. Blaser, A. Indolese, A. Schnyder, *Curr. Sci.* 78 (2000) 1336–1344.
- [21] D.J. Cole-Hamilton, *Science* 299 (2003) 1702–1706.
- [22] D. Ager, *Handbook of Chiral Chemicals*, Taylor & Francis, Boca Raton, 2005.
- [23] L. Yin, W. Shan, X. Jia, X. Li, A.S.C. Chan, *J. Organomet. Chem.* 694 (2009) 2092–2095.
- [24] S. Salunke, A. Paul, R. Nair, R.V. Kintali, *Process for the Preparation of Clopidogrel Bisulphate Form I*, WO2009080469 A1, 2009.
- [25] D.J. Ager, *Platin. Met. Rev.* 53 (2009) 203–208.
- [26] P.-C. Yan, G.-L. Zhu, J.-H. Xie, X.-D. Zhang, Q.-L. Zhou, Y.-Q. Li, W.-H. Shen, D.-Q. Che, *Org. Process Res. Dev.* 17 (2013) 307–312.
- [27] M. Breuer, K. Ditrich, T. Habicher, B. Hauer, M. Keßeler, R. Stürmer, T. Zelinski, *Angew. Chemie - Int. Ed.* 43 (2004) 788–824.
- [28] R.W. Stringham, Y.K. Ye, *J. Chromatogr. A* 1101 (2006) 86–93.
- [29] Q. He, S. Rohani, J. Zhuh, A. Gomaa, *Chirality* 24 (2012) 119–128.
- [30] K. Vukics, T. Fodor, J. Fischer, I. Fellegvari, S. Levai, *Org.*

Process Res. Dev. 6 (2002) 82–85.

- [31] B.M. Khamar, I.A. Modi, Process for the Preparation of Sertraline Hydrochloride Form II, WO 2006027658 A2, 2006.
- [32] P.J. Dunn, A.S. Wells, M.T. Williams, Green Chemistry in the Pharmaceutical Industry, Wiley-VCH, Mörlenbach, 2010.
- [33] K.E. Jaeger, Curr. Opin. Biotechnol. 15 (2004) 269–271.
- [34] R.C. Simon, N. Richter, E. Busto, W. Kroutil, ACS Catal. 4 (2014) 129–143.
- [35] F. Rudroff, M.D. Mihovilovic, H. Gröger, R. Snajdrova, H. Iding, U.T. Bornscheuer, Nat. Catal. 1 (2018) 12–22.
- [36] A. Schmid, F. Hollmann, J.B. Park, B. Bühler, Curr. Opin. Biotechnol. 13 (2002) 359–366.
- [37] A. Schmid, J.S. Dordick, B. Hauer, A. Kiener, M. Wubbolt, B. Witholt, Nature 409 (2001) 258–268.
- [38] N.J. Weise, F. Parmeggiani, S.T. Ahmed, N.J. Turner, J. Am. Chem. Soc. 137 (2015) 12977–12983.
- [39] T. Knaus, W. Böhmer, F.G. Mutti, Green Chem. 19 (2017) 453–463.
- [40] J.A. McIntosh, P.S. Coelho, C.C. Farwell, Z.J. Wang, J.C. Lewis, T.R. Brown, F.H. Arnold, Angew. Chemie - Int. Ed. 52 (2013) 9309–9312.
- [41] E. O'Reilly, N.J. Turner, Perspect. Sci. 4 (2015) 55–61.
- [42] I. Rowles, K.J. Malone, L.L. Etchells, S.C. Willies, N.J. Turner, ChemCatChem 4 (2012) 1259–1261.
- [43] R.A. John, Biochim. Biophys. Acta 1248 (1995) 81–96.
- [44] F. Guo, P. Berglund, Green Chem. 19 (2017) 333–360.
- [45] T. Lütke-Eversloh, G. Stephanopoulos, Metab. Eng. 10 (2008) 69–77.
- [46] W.D. Fessner, N. Biotechnol. 32 (2015) 658–664.

References

- [47] P.K. Mehta, T.I. Hale, P. Christen, *Eur. J. Biochem.* 561 (1993) 549–561.
- [48] S. Mathew, H. Yun, *ACS Catal.* 2 (2012) 993–1001.
- [49] A. Iwasaki, Y. Yamada, N. Kizaki, Y. Ikenaka, J. Hasegawa, *Appl. Microbiol. Biotechnol.* 69 (2006) 499–505.
- [50] A. Iwasaki, K. Matsumoto, J. Hasegawa, Y. Yasohara, *Appl. Microbiol. Biotechnol.* 93 (2012) 1563–1573.
- [51] L.-J. Guan, J. Ohtsuka, M. Okai, T. Miyakawa, T. Mase, Y. Zhi, F. Hou, N. Ito, A. Iwasaki, Y. Yasohara, M. Tanokura, *Sci. Rep.* 5 (2015) 10753.
- [52] M. Girardin, S.G. Ouellet, D. Gauvreau, J.C. Moore, G. Hughes, P.N. Devine, P.D. O'Shea, L.C. Campeau, *Org. Process Res. Dev.* 17 (2013) 61–68.
- [53] C. Molinaro, P.G. Bulger, E.E. Lee, B. Kosjek, S. Lau, D. Gauvreau, M.E. Howard, D.J. Wallace, P.D. O'Shea, *J. Org. Chem.* 77 (2012) 2299–2309.
- [54] Sebastian Schätzle, *Identification, Characterization and Application of Novel (R)-Selective Amine Transaminases*, Ernst-Moritz-Arndt-Universität Greifswald, 2011.
- [55] A. Mozzarelli, S. Bettati, *Chem. Rec.* 6 (2006) 275–287.
- [56] L. Rios-Solis, N. Bayir, M. Halim, C. Du, J.M. Ward, F. Baganz, G.J. Lye, *Biochem. Eng. J.* 73 (2013) 38–48.
- [57] D. Koszelewski, K. Tauber, K. Faber, W. Kroutil, *Trends Biotechnol.* 28 (2010) 324–332.
- [58] K.E. Cassimjee, B. Manta, F. Himo, *Org. Biomol. Chem.* 13 (2015) 8453–8464.
- [59] K.E. Cassimjee, M.S. Humble, V. Miceli, C.G. Colomina, P. Berglund, *ACS Catal.* 1 (2011) 1051–1055.
- [60] E. Krissinel, K. Henrick, *J. Mol. Biol.* 372 (2007) 774–797.
- [61] B. Bjellqvist, B. Basse, E. Olsen, J.E. Celis, *Electrophoresis* 15 (1994) 529–539.

-
- [62] B. Bjellqvist, G.J. Hughes, C. Pasquali, N. Paquet, F. Ravier, J.-C. Sanchez, S. Frutiger, D. Hochstrasser, *Electrophoresis* 14 (1993) 1023–1031.
- [63] M.R. Wilkins, E. Gasteiger, A. Bairoch, J. Sanchez, K.L. Williams, R.D. Appel, D.F. Hochstrasser, in: *Methods Mol. Biol.*, A.J. Link, Totowa, 2005, pp. 531–552.
- [64] J.S. Shin, B.G. Kim, *Biotechnol. Bioeng.* 65 (1999) 206–11.
- [65] M.D. Truppo, J.D. Rozzell, N.J. Turner, *Org. Process Res. Dev.* 14 (2010) 234–237.
- [66] D. Koszelewski, D. Clay, D. Rozzell, W. Kroutil, *European J. Org. Chem.* (2009) 2289–2292.
- [67] P. Tufvesson, J. Lima-Ramos, J.S. Jensen, N. Al-Haque, W. Neto, J.M. Woodley, *Biotechnol. Bioeng.* 108 (2011) 1479–1493.
- [68] M. D. Patil, G. Grogan, A. Bommarius, H. Yun, *Catalysts* 8 (2018) 2073–4344.
- [69] H. Yun, B.Y. Hwang, J.H. Lee, B.G. Kim, *Appl. Environ. Microbiol.* 71 (2005) 4220–4224.
- [70] D. Koszelewski, M. Göritzer, D. Clay, B. Seisser, W. Kroutil, *ChemCatChem* 2 (2010) 73–77.
- [71] M. Planchestainer, M.L. Contente, J. Cassidy, F. Molinari, L. Tamborini, F. Paradisi, *Green Chem.* 19 (2017) 372–375.
- [72] M.D. Truppo, J.D. Rozzell, J.C. Moore, N.J. Turner, *Org. Biomol. Chem.* 7 (2009) 395–398.
- [73] L. Rios-Solis, P. Morris, C. Grant, A.O.O. Odeleye, H.C. Hailes, J.M. Ward, P.A. Dalby, F. Baganz, G.J. Lye, *Chem. Eng. Sci.* 122 (2015) 360–372.
- [74] E.S. Park, J.Y. Dong, J.S. Shin, *Org. Biomol. Chem.* 11 (2013) 6929–6933.
- [75] R.E. Meadows, K.R. Mulholland, M. Schürmann, M. Golden, H. Kierkels, E. Meulenbroeks, D. Mink, O. May, C. Squire, H. Straatman, A.S. Wells, *Org. Process Res. Dev.* 17 (2013)

References

1117–1122.

- [76] J.-S. Shin, B.-G. Kim, D.-H. Shin, *Enzyme Microb. Technol.* 29 (2001) 232–239.
- [77] J.S. Shin, B.G. Kim, *Biotechnol. Bioeng.* 77 (2002) 832–837.
- [78] B.K. Cho, H.J. Cho, H. Yun, B.G. Kim, *J. Mol. Catal. B Enzym.* 26 (2003) 273–285.
- [79] A.P. Green, N.J. Turner, E. O'Reilly, *Angew. Chemie - Int. Ed.* 53 (2014) 10714–10717.
- [80] H.H. Lo, S.K. Hsu, W. De Lin, N.L. Chan, W.H. Hsu, *Biotechnol. Prog.* 21 (2005) 411–415.
- [81] D. Koszelewski, I. Lavandera, D. Clay, D. Rozzell, W. Kroutil, *Adv. Synth. Catal.* 350 (2008) 2761–2766.
- [82] F.G. Mutti, C.S. Fuchs, D. Pressnitz, J.H. Sattler, W. Kroutil, *Adv. Synth. Catal.* 353 (2011) 3227–3233.
- [83] M. Höhne, S. Köhl, K. Robins, U.T. Bornscheuer, *ChemBioChem* 9 (2008) 363–365.
- [84] D. Koszelewski, I. Lavandera, D. Clay, G.M. Guebitz, D. Rozzell, W. Kroutil, *Angew. Chemie - Int. Ed.* 47 (2008) 9337–9340.
- [85] K.E. Cassimjee, C. Branneby, V. Abedi, A. Wells, P. Berglund, *Chem. Commun.* 46 (2010) 5569.
- [86] S. Schätzle, F. Steffen-Munsberg, A. Thontowi, M. Höhne, K. Robins, U.T. Bornscheuer, *Adv. Synth. Catal.* 353 (2011) 2439–2445.
- [87] Y. Li, S. Jing, C. Cuijie, W. Shaoming, Z. Liande, X. Wenlin, G. Liping, *Electrophoresis* 30 (2009) 3527–3533.
- [88] S. Pedragosa-Moreau, A. Le Flohic, V. Thienpondt, F. Lefoulon, A.M. Petit, N. Ríos-Lombarda, F. Moras, J. Gonzalez-Saban, *Adv. Synth. Catal.* 359 (2017) 485–493.
- [89] U.T. Bornscheuer, *Angew. Chemie - Int. Ed.* 42 (2003) 3336–3337.

-
- [90] R. Ye, J. Zhao, B.B. Wickemeyer, F.D. Toste, G.A. Somorjai, *Nat. Catal.* (2018).
- [91] U. Hanefeld, L. Gardossi, E. Magner, *Chem. Soc. Rev.* 38 (2009) 453–468.
- [92] F. Rigoldi, S. Donini, A. Redaelli, E. Parisini, A. Gautieri, *APL Bioeng.* 2 (2018) 011501.
- [93] R.A. Sheldon, S. van Pelt, *Chem. Soc. Rev.* 42 (2013) 6223–6235.
- [94] G. Fernández-Lorente, M. Terreni, C. Mateo, A. Bastida, R. Fernández-Lafuente, P. Dalmases, J. Huguët, J.M. Guisán, *Enzyme Microb. Technol.* 28 (2001) 389–396.
- [95] N. Khalaf, C.P. Govardhan, J.J. Lalonde, R.A. Persichetti, Y.F. Wang, A.L. Margolin, *J. Am. Chem. Soc.* 118 (1996) 5494–5495.
- [96] C. Mateo, J.M. Palomo, G. Fernandez-Lorente, J.M. Guisan, R. Fernandez-Lafuente, *Enzyme Microb. Technol.* 40 (2007) 1451–1463.
- [97] L. Cao, F. van Rantwijk, R.A. Sheldon, *Org. Lett.* 2 (2000) 1361–1364.
- [98] R.A. Sheldon, *Adv. Synth. Catal.* 349 (2007) 1289–1307.
- [99] L. Cao, *Carrier-Bound Immobilized Enzymes*, John Wiley & Sons, Weinheim, 2006.
- [100] F. Jia, B. Narasimhan, S. Mallapragada, *Biotechnol. Bioeng.* 111 (2014) 209–222.
- [101] D. Gaffney, J. Cooney, E. Magner, *Top. Catal.* 55 (2012) 1101–1106.
- [102] S. Duinhoven, R. Poort, G. Vandervoet, W.G.M. Agterof, W. Norde, J. Lyklema, *J. Colloid Interface Sci.* 170 (1995) 340–350.
- [103] W. Norde, W. Tan, L. Koopal, *Rev. La Cienc. Del Suelo y Nutr. Veg.* 8 (2008).

References

- [104] Y.X. Bai, Y.F. Li, Y. Yang, L.X. Yi, *Process Biochem.* 41 (2006) 770–777.
- [105] D.N. Tran, K.J. Balkus, *ACS Catal.* 1 (2011) 956–968.
- [106] J. Pedroche, M. del Mar Yust, C. Mateo, R. Fernández-Lafuente, J. Girón-Calle, M. Alaiz, J. Vioque, J.M. Guisán, F. Millán, *Enzyme Microb. Technol.* 40 (2007) 1160–1166.
- [107] T. Jesionowski, J. Zdarta, B. Krajewska, *Adsorption* 20 (2014) 801–821.
- [108] V. Singh, P. Kumar, *J. Mol. Catal. B Enzym.* 70 (2011) 67–73.
- [109] S.K. Shukla, A.K. Mishra, O.A. Arotiba, B.B. Mamba, *Int. J. Biol. Macromol.* 59 (2013) 46–58.
- [110] H. Mallin, U. Menyes, T. Vorhaben, M. Höhne, U.T. Bornscheuer, *ChemCatChem* 5 (2013) 588–593.
- [111] S.S. Yi, C. won Lee, J. Kim, D. Kyung, B.G. Kim, Y.S. Lee, *Process Biochem.* 42 (2007) 895–898.
- [112] H. Mallin, M. Höhne, U.T. Bornscheuer, *J. Biotechnol.* 191 (2014) 32–37.
- [113] C. Bernal, A. Illanes, L. Wilson, *Langmuir* 30 (2014) 3557–3566.
- [114] V. Smeets, L. Ben Mustapha, J. Schnee, E.M. Gaigneaux, D.P. Debecker, *Mol. Catal.* 452 (2018) 123–128.
- [115] I. Migneault, C. Dartiguenave, M.J. Bertrand, K.C. Waldron, *Biotechniques* 37 (2004) 790–802.
- [116] R.F. Borch, M.D. Bernstein, H.D. Durst, *J. Am. Chem. Soc.* 93 (1971) 2897–2904.
- [117] D. Koszelewski, N. Müller, J.H. Schrittwieser, K. Faber, W. Kroutil, *J. Mol. Catal. B Enzym.* 63 (2010) 39–44.
- [118] J.J. Roy, T.E. Abraham, *Chem. Rev.* 104 (2004) 3705–3721.
- [119] R.A. Sheldon, R. Schoevaart, L.M. Van Langen, *Biocatal. Biotransformation* 23 (2005) 141–147.

-
- [120] J. Zdarta, A. Meyer, T. Jesionowski, M. Pinelo, *Catalysts* 8 (2018) 92.
- [121] Y. Lü, G. Lu, Y. Wang, Y. Guo, Y. Guo, Z. Zhang, Y. Wang, X. Liu, *Adv. Funct. Mater.* 17 (2007) 2160–2166.
- [122] E. Katchalski-Katzir, D.M. Kraemer, *J. Mol. Catal. - B Enzym.* 10 (2000) 157–176.
- [123] N. Brun, A. Babeau Garcia, H. Deleuze, M.F. Achard, C. Sanchez, F. Durand, V. Oestreicher, R. Backov, *Chem. Mater.* 22 (2010) 4555–4562.
- [124] M.D. Truppo, H. Strotman, G. Hughes, *ChemCatChem* 4 (2012) 1071–1074.
- [125] D.K. Gale, T. Cutu, J. Jiao, C.H. Chang, G.L. Rorrer, *Adv. Funct. Mater.* 19 (2009) 926–933.
- [126] M. Niederberger, N. Pinna, *Metal Oxide Nanoparticles in Organic Solvents*, Springer, London, 2009.
- [127] C.J. Brinker, G.W. Scherer, *Sol-Gel Science*, Academic Press, Boston, 1990.
- [128] D.P. Debecker, *Chem. Rec.* 18 (2018) 662–675.
- [129] D.P. Debecker, S. Le Bras, C. Boissière, A. Chaumonnot, C. Sanchez, *Chem. Soc. Rev.* 47 (2018) 4112–4155.
- [130] Z. Zhou, M. Hartmann, *Chem. Soc. Rev.* 42 (2013) 3894–3912.
- [131] N. Carlsson, H. Gustafsson, C. Thörn, L. Olsson, K. Holmberg, B. Åkerman, *Adv. Colloid Interface Sci.* 205 (2014) 339–360.
- [132] Y. Wang, F. Caruso, (2005) 953–961.
- [133] N. Brun, A. Babeau-Garcia, M.-F. Achard, C. Sanchez, F. Durand, G. Laurent, M. Birot, H. Deleuze, R. Backov, *Energy Environ. Sci.* 4 (2011) 2840.
- [134] Liu, Rieben, Iversen, Sørensen, Park, Nygård, K. Martinez, *Nanotechnology* 21 (2010) 245105.

References

- [135] S.-W. Xu, Y. Lu, J. Li, Y.-F. Zhang, Z.-Y. Jiang, J. Biomater. Sci. - Polym. Ed. 18 (2007) 71–80.
- [136] L.T. Zhuravlev, Colloids Surfaces A Physicochem. Eng. Asp. 173 (2000) 1–38.
- [137] K.M.R. Kallury, P.M. Macdonald, M. Thompson, Langmuir 10 (1994) 492–499.
- [138] C. Sanchez, F. Ribot, L. Rozes, B. Alonso, New J. Chem. 18 (1994) 1007–1047.
- [139] E.T. Vandenberg, L. Bertilsson, B. Liedberg, K. Uvdal, R. Erlandsson, H. Elwing, I. Lundström, J. Colloid Interface Sci. 147 (1991) 103–118.
- [140] M. Etienne, A. Walcarius, Talanta 59 (2003) 1173–1188.
- [141] C. Jaroniec, M. Kruk, J. Phys. Chem. B 102 (1998) 5503–5510.
- [142] T. Kovalchuk, H. Sfihi, L. Kostenko, V. Zaitsev, J. Fraissard, J. Colloid Interface Sci. 302 (2006) 214–229.
- [143] P.P. Luan, Y.J. Jiang, S.P. Zhang, J. Gao, Z.G. Su, G.H. Ma, Y.F. Zhang, J. Biosci. Bioeng. 118 (2014) 575–582.
- [144] E. Guibal, Prog. Polym. Sci. 30 (2005) 71–109.
- [145] K. Molvinger, F. Quignard, D. Brunel, M. Boissière, J.M. Devoisselle, Chem. Mater. 16 (2004) 3367–3372.
- [146] N.R. Kildeeva, P.A. Perminov, L. V. Vladimirov, V. V. Novikov, S.N. Mikhailov, Russ. J. Bioorganic Chem. 35 (2009) 360–369.
- [147] K. Engelmark Cassimjee, M. Kadow, Y. Wikmark, M. Svedendahl Humble, M.L. Rothstein, D.M. Rothstein, J.-E. Bäckvall, Chem. Commun. 50 (2014) 9134.
- [148] M. Päiviö, L.T. Kanerva, Process Biochem. 48 (2013) 1488–1494.
- [149] I. Patramani, Eur. J. Biochem. 11 (1969) 28–36.
- [150] G. Jas, A. Kirschning, Chem. - A Eur. J. 9 (2003) 5708–5723.

-
- [151] A. Kirschning, W. Solodenko, K. Mennecke, *Chem. - A Eur. J.* 12 (2006) 5972–5990.
- [152] M.B. Plutschack, B. Pieber, K. Gilmore, P.H. Seeberger, *Chem. Rev.* 117 (2017) 11796–11893.
- [153] R. Yuryev, S. Strompen, A. Liese, *Beilstein J. Org. Chem.* 7 (2011) 1449–1467.
- [154] T. Honda, M. Miyazaki, Y. Yamaguchi, H. Nakamura, H. Maeda, *Lab Chip* 7 (2007) 366–372.
- [155] S. Mitchell, N.-L. Michels, J. Pérez-Ramírez, *Chem. Soc. Rev.* 42 (2013) 6094.
- [156] K. Engström, E. V. Johnston, O. Verho, K.P.J. Gustafson, M. Shakeri, C.W. Tai, J.E. Bäckvall, *Angew. Chemie - Int. Ed.* 52 (2013) 14006–14010.
- [157] S. Ungureanu, G. Laurent, H. Deleuze, O. Babot, M.F. Achard, M.I. Popa, C. Sanchez, R. Backov, *Colloids Surfaces A Physicochem. Eng. Asp.* 360 (2010) 85–93.
- [158] D.P. Debecker, C. Boissière, G. Laurent, S. Huet, P. Eliaers, C. Sanchez, R. Backov, *Chem. Commun.* 51 (2015) 14018–14021.
- [159] S. Matosevic, G.J. Lye, F. Baganz, *J. Biotechnol.* 155 (2011) 320–329.
- [160] A. Abdul Halim, N. Szita, F. Baganz, *J. Biotechnol.* 168 (2013) 567–575.
- [161] L.H. Andrade, W. Kroutil, T.F. Jamison, *Org. Lett.* 16 (2014) 6092–6095.
- [162] G. Rehn, P. Adlercreutz, C. Grey, *J. Biotechnol.* 179 (2014) 50–55.
- [163] M. Bajić, I. Plazl, R. Stloukal, P. Žnidaršič-Plazl, *Process Biochem.* 52 (2017) 63–72.
- [164] M.L. Contente, F. Paradisi, *Nat. Catal.* (2018) 1.
- [165] R.L. Hanson, B.L. Davis, Y. Chen, S.L. Goldberg, W.L. Parker,

References

- T.P. Tully, M.A. Montana, R.N. Patel, *Adv. Synth. Catal.* 350 (2008) 1367–1375.
- [166] S. Ungureanu, M. Birot, G. Laurent, H. Deleuze, O. Babot, B. Julián-López, M.F. Achard, M.I. Popa, C. Sanchez, R. Backov, *Chem. Mater.* 19 (2007) 5786–5796.
- [167] T. Endo, S. Okada, K. Torigoe, N. Koshikawa, R.G. Shresta, K. Sakai, S. Koura, M. Abe, H. Sakai, *Colloids Surfaces A Physicochem. Eng. Asp.* 464 (2015) 52–56.
- [168] J. Kirchnerova, G.C.B. Cave, *Can J Chem* 54 (1976) 3909–3916.
- [169] M. Mihailović, M. Stojanović, K. Banjanac, M. Carević, N. Prlainović, N. Milosavić, D. Bezbradica, *Process Biochem.* 49 (2014) 637–646.
- [170] M.M. Bradford, *Anal. Biochem.* 72 (1976) 248–254.
- [171] T. Zor, Z. Selinger, *Anal. Biochem.* 236 (1996) 302–308.
- [172] A. Chang, I. Schomburg, S. Placzek, L. Jeske, M. Ulbrich, M. Xiao, C.W. Sensen, D. Schomburg, *Nucleic Acids Res.* 43 (2015) D439–D446.
- [173] C.J. Brinker, G.W. Scherer, *Sol-Gel Science: The Physics and Chemistry of Sol-Gel Processing*, Academic Press, 1990.
- [174] A.M. Putz, M. V. Putz, *Int. J. Mol. Sci.* 13 (2012) 15925–15941.
- [175] R.M. Pasternack, S.R. Amy, Y.J. Chaba, *Langmuir* 24 (2008) 12963–12971.
- [176] A.N. Kursunlu, E. Guler, H. Dumrul, O. Kocyigit, I.H. Gubbuk, *Appl. Surf. Sci.* 255 (2009) 8798–8803.
- [177] T.P. Mollart, K.L. Lewis, C.S.J. Pickles, C.J.H. Wort, *Semicond. Sci. Technol.* 18 (2003) S117–S124.
- [178] Y.-X. Bai, Y.-F. Li, Y. Yang, L.-X. Yi, *Process Biochem.* 41 (2006) 770–777.
- [179] Z. Hu, L. Xu, X. Wen, *J. Environ. Sci. (China)* 25 (2013) 181–187.

-
- [180] P. Tufvesson, J.S. Jensen, W. Kroutil, J.M. Woodley, *Biotechnol. Bioeng.* 109 (2012) 2159–2162.
- [181] P. Tufvesson, C. Bach, J.M. Woodley, *Biotechnol. Bioeng.* 111 (2014) 309–319.
- [182] A. Calvo, P.C. Angelomé, V.M. Sánchez, D.A. Scherlis, F.J. Williams, G.J.A.A. Soler-Illia, *Chem. Mater.* 20 (2008) 4661–4668.
- [183] F. Carn, A. Colin, M.-F. Achard, H. Deleuze, E. Sellier, M. Birot, R. Backov, *J. Mater. Chem.* 14 (2004) 1370–1376.
- [184] N. Gartmann, C. Schütze, H. Ritter, D. Brühwiler, *J. Phys. Chem. Lett.* 1 (2010) 379–382.
- [185] O. Barbosa, R. Torres, C. Ortiz, R. Fernandez-Lafuente, *Process Biochem.* 47 (2012) 1220–1227.
- [186] S.K. Shukla, A.K. Mishra, O.A. Arotiba, B.B. Mamba, *Int. J. Biol. Macromol.* 59 (2013) 46–58.
- [187] E.P. Cipolatti, M.J.A. Silva, M. Klein, V. Feddern, M.M.C. Feltes, J.V. Oliveira, J.L. Ninow, D. De Oliveira, *J. Mol. Catal. B Enzym.* 99 (2014) 56–67.
- [188] S. Bhuvaneshwari, D. Sruthi, V. Sivasubramanian, Niranjana kalyani, J. Sugunabai, *Int. J. Eng. Res. Appl.* 1 (2011) 292–299.
- [189] E. Guibal, *Sep. Purif. Technol.* 38 (2004) 43–74.
- [190] A.A. Khan, S. Akhtar, Q. Husain, *J. Mol. Catal. B Enzym.* 40 (2006) 58–63.
- [191] Privalov P.L., *Crit. Rev. Biochem. Mol. Biol.* 25 (1990) 281–305.
- [192] R.F. Borch, M.D. Bernstein, H.D. Durst, *J. Am. Chem. Soc.* 93 (1971) 2897–2904.
- [193] E.T. Hwang, M.B. Gu, *Eng. Life Sci.* 13 (2013) 49–61.
- [194] J. Ma, L. Zhang, Z. Liang, W. Zhang, Y. Zhang, *J. Sep. Sci.* 30 (2007) 3050–3059.

References

- [195] B. Golinelli-pimpaneau, B. Badet, *Eur. J. Biochem.* 201 (1991) 175–182.
- [196] C. López, S.D. Ríos, J. López-Santín, G. Caminal, G. Álvaro, *Biochem. Eng. J.* 49 (2010) 414–421.
- [197] C.D. Nunes, J. Pires, A.P. Carvalho, M.J. Calhorda, P. Ferreira, *Microporous Mesoporous Mater.* 111 (2008) 612–619.
- [198] J. Pires, M. Pinto, J. Estella, J.C. Echeverría, *J. Colloid Interface Sci.* 317 (2008) 206–213.
- [199] M. Petkar, A. Lali, P. Caimi, M. Daminati, *J. Mol. Catal. B Enzym.* 39 (2006) 83–90.
- [200] M. Rondon-Gonzalez, V. Sadtler, L. Choplin, J.-L. Salager, *Colloids Surfaces A Physicochem. Eng. Asp.* 288 (2006) 151–157.
- [201] Liu, Rieben, Iversen, Sørensen, Park, Nygård, K. Martinez, *Nanotechnology* 21 (2010) 245105.
- [202] P. Vejayakumaran, I.A. Rahman, C.S. Sipaut, J. Ismail, C.K. Chee, *J. Colloid Interface Sci.* 328 (2008) 81–91.
- [203] J.A. Bornhorst, J.J. Falke, *Methods Enzym.* 326 (2000) 245–254.
- [204] J.P. Guthrie, *Bioorg. Chem.* 30 (2002) 32–52.
- [205] R.W. Hay, M.A. Bond, *Aust. J. Chem.* 20 (1967) 1823–1828.
- [206] J.P. Guthrie, F. Jordan, *J. Am. Chem. Soc.* 94 (1972) 9136–9141.
- [207] G.J. van Stekelenburg, G. Koorevaar, *Clin. Chim. Acta* 39 (1972) 191–199.
- [208] M.D. Truppo, in: *Diabetes*, 2009, pp. 1–23.
- [209] M. Fuchs, D. Koszelewski, K. Tauber, W. Kroutil, K. Faber, *Chem. Commun.* 46 (2010) 5500.
- [210] D. Koszelewski, N. Müller, J.H. Schrittwieser, K. Faber, W.

- Kroutil, J. *Mol. Catal. B Enzym.* 63 (2010) 39–44.
- [211] V. Leskovac, S. Trivić, D. Peričin, M. Popović, J. Kandrač, J. Serbian Chem. Soc. 75 (2010) 185–194.
- [212] F.M. Dickinson, G.P. Monger, *Biochem. J.* 131 (1973) 261–270.
- [213] C.B. Jendresen, S.G. Stahlhut, M. Li, P. Gaspar, S. Siedler, J. Förster, J. Maury, I. Borodina, A.T. Nielsen, *Appl. Environ. Microbiol.* 81 (2015) 4458–4476.
- [214] S. Pilbák, Ö. Farkas, L. Poppe, *Chem. - A Eur. J.* 18 (2012) 7793–7802.
- [215] K.T. Watts, B.N. Mijts, P.C. Lee, A.J. Manning, C. Schmidt-Dannert, *Chem. Biol.* 13 (2006) 1317–1326.
- [216] J.A. Kyndt, T.E. Meyer, M.A. Cusanovich, J.J. Van Beeumen, *Febs Lett* 512 (2002) 240–244.
- [217] F. Parmeggiani, N.J. Weise, S.T. Ahmed, N.J. Turner, *Chem. Rev.* 118 (2018) 73–118.
- [218] A.C. Schroeder, S. Kumaran, L.M. Hicks, R.E. Cahoon, C. Halls, O. Yu, J.M. Jez, *Phytochemistry* 69 (2008) 1496–1506.
- [219] S. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, *J. Mol. Biol.* 215 (1990) 403–410.
- [220] S. Bartsch, U.T. Bornscheuer, *Angew. Chemie - Int. Ed.* 48 (2009) 3362–3365.
- [221] S. Bettati, S. Benci, B. Campanini, S. Raboni, G. Chirico, S. Beretta, K.D. Schnackerz, T.L. Hazlett, E. Gratton, A. Mozzarelli, *J. Biol. Chem.* 275 (2000) 40244–40251.
- [222] M.S. Malik, E.S. Park, J.S. Shin, *Appl. Microbiol. Biotechnol.* 94 (2012) 1163–1171.
- [223] M. Svedendahl, *Lipase and ω -Transaminase – Biocatalytic Investigations*, 2010.
- [224] W. Neto, M. Schürmann, L. Panella, A. Vogel, J.M. Woodley, *J. Mol. Catal. B Enzym.* 117 (2015) 54–61.

References

- [225] K.E. Cassimjee, C. Branneby, V. Abedi, A. Wells, P. Berglund, *Chem. Commun.* 46 (2010) 5569.
- [226] J.H. Jensen, J.C. Kromann, *J. Chem. Educ.* 90 (2013) 1093–1095.

Annexes

1 ATA-117 – batch investigations

1.1 Determining the kinetic parameters

Kinetic parameters of ATA-117 Transaminase have been measured in order to assess the biocatalytic efficiency under different conditions. These parameters are related to the Michaelis-Menten kinetic equation, below (where $[S]$ is the substrate concentration, t is the time, k_{CAT} is the rate constant of the catalytic act, $[E]_t$ is the total enzyme concentration, and K_m is the Michaelis-Menten constant (related to the affinity of the activated enzyme for the substrate)).

$$\frac{-\partial[S]}{\partial t} = v = \frac{k_{CAT}[E]_t[S]}{K_m + [S]}$$

In our case, the enzyme is not saturated by the substrate (see Annexe 1.5) so that the substrate concentration $[S]$ is neglected regarding to the Michaelis constant K_m ($K_m \gg [S]$). The rate is then governed by the substrate concentration and a global rate constant named k_{OBS} . In the following, k_{OBS} is given as a measure of enzymatic activity, as $[S]$ was kept constant (unless stated otherwise).

$$v = \frac{k_{CAT} \cdot [E]_t}{K_m} \cdot [S] = k_{OBS} \cdot [S]$$

The parameter k_{OBS} is assessed by measuring the time needed to convert half of the substrate, $t_{1/2}$, given by the integration of the Michaelis-Menten equation.

$$k_{OBS} \int_0^{t_{1/2}} \partial t = - \int_{[S]}^{\frac{[S]}{2}} \frac{1}{[S]} \cdot \partial [S]$$

$$k_{OBS} [t_{1/2} - 0] = -[\ln([S]/2) - \ln([S])]$$

$$k_{OBS} t_{1/2} = \ln(2)$$

For example, in the best preparation and pH conditions (see Annexe 1.2), the transaminase ATA-117 converts 50 % of R-BMBA in 30 minutes:

$$k_{OBS} = \frac{\ln(2)}{t_{1/2}} = \frac{0.69}{30} = 0.023 \text{ min}^{-1}$$

This experiment was performed with $[E]_t = 4.4 \text{ } \mu\text{M}$ transaminase, allowing to calculate the catalytic efficiency, *i.e.* the ratio of the catalytic rate constant (k_{CAT}) over the Michaelis-Menten constant (K_m):

$$\frac{k_{CAT}}{K_m} = \frac{k_{OBS}}{[E]_t} = \frac{0.023}{4.4 \cdot 10^{-6}} = 5.2 \cdot 10^3 \text{ M}^{-1} \text{ min}^{-1}$$

This ratio is quite low as compared to other transaminases that reach $10^7 \text{ M}^{-1} \cdot \text{min}^{-1}$ [172].

1.2 ATA-117 solution preparation

In order to use the transaminase ATA-117, one must solubilize the lyophilized powder into a buffer. The presence of the PLP cofactor and the sodium pyruvate in the phosphate buffer used for the solubilisation had an influence over the catalytic activity (Figure A 1, A and C). Indeed, in the case A, the powder was solubilized in the phosphate buffer (pH 8, 100 mM) and then added in the batch reactors containing the reaction medium. In the case C, the enzyme powder was solubilized in a buffer containing PLP (0.5 g/L, that is 2.02 mM) and sodium pyruvate (10 mM) in phosphate buffer (pH 8, 100 mM). The presence of PLP and pyruvate since the beginning of the solubilisation process showed clearly a positive effect on the activity (through k_{OBS} values). The PLP influence on enzyme stability has been reported for other PLP-dependent enzymes [221] .

The storage stability on these conditions has been studied (Figure A 1, B and D). The activity of the solutions B and D (respectively solubilized in phosphate buffer only, and in phosphate buffer with pyruvate and PLP) have been measured after 24 h of cold storage (4 °C). A deactivation was observed in both cases (an activity loss of 33 % is observed for the solution prepared in phosphate buffer only, and of 25 % for the solution prepared in phosphate buffer with PLP and pyruvate).

In further experiments, the ATA-117 transaminase powder was always solubilized in the phosphate buffer containing PLP and sodium pyruvate, and used directly without storage, unless stated otherwise.

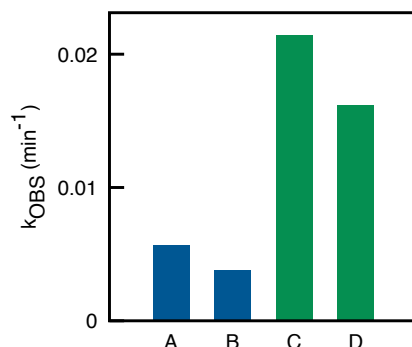


Figure A 1 – Effect of solubilisation buffer composition and solution storage stability. (A) The powder was solubilized in the phosphate buffer, (B) and eventually stored for 24 h at 4 °C, prior to the batch reaction. (C) The powder was solubilized in the phosphate buffer in the presence of PLP and pyruvate, (D) and eventually stored for 24 h at 4 °C, prior to the batch reaction. The presence of PLP and pyruvate in the solubilisation buffer had a positive effect on enzyme activity. The cold storage of transaminase solutions caused a deactivation. ATA-117 concentration was 4.4 μM in each batches. RSD (Relative standard deviation) is 3 %.

1.3 ATA-117 extended storage stability

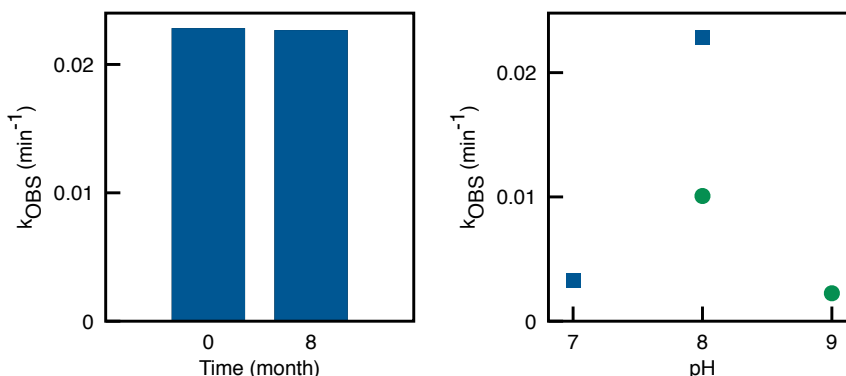


Figure A 2 – Storage and pH stability of ATA-117. (Left) ATA-117 extended storage stability (lyophilized powder at -20 °C). No significant deactivation was observed after 8 months of storage. ATA-117 concentration was 4.4 μM in each batches. RSD is 3 %. (Right) ATA-117 pH optimization in phosphate buffer (■) or in borate buffer (●). The optimum pH for preparation and reaction was pH 8 with phosphate buffer. ATA-117 concentration was 4.4 μM in each batches. RSD is 3 %.

The stability of ATA-117 transaminase over an extended storage at -20 °C (lyophilized powder) was measured over time (Figure A 2, left). After an 8-month period, no significant deactivation was

observed indicating that this extended cold storage method was reliable.

1.4 ATA-117 pH optimization

Optimum pH of ATA-117 has been checked. ATA-117 transaminase solutions have been prepared in different pH buffers, and the activities were checked for those pH, respectively in phosphate buffer of pH 7 and 8, and in borate buffer of pH 8 and 9 (Figure A 2, right). Not surprisingly, the highest activity was observed for the enzyme solubilized in phosphate buffer of pH 8. The use of pH 8 borate buffer for solubilisation and reaction led to a halved activity. A more acidic or alkaline buffer leads to a drop of activity.

1.5 Kinetic parameters for ATA-117

In a previous work (master thesis), substrate concentrations have been varied in order to determine kinetic parameters for ATA-117 (homogeneous batch reactors, Figure A 3).

It was observed that, by changing rac-BMBA concentrations, activity reached a maximum before dropping. Rac-BMBA is then inhibiting ATA-117 at high concentration levels (above 10 mM). This substrate inhibition is widely reported in the literature [79,222–224]. This inhibition was not observed for high sodium pyruvate concentrations (up to 50 mM).

In order to maximise enzyme activity, a rac-BMBA concentration of 10 mM was chosen. In these conditions, the enzyme is not fully saturated by its substrate.

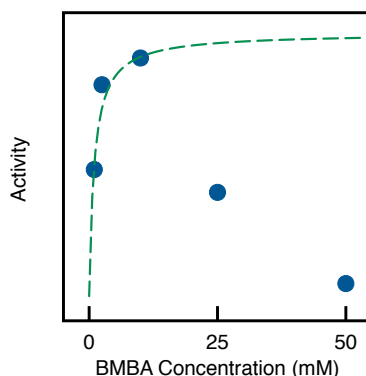


Figure A 3 – Activity versus rac-BMBA concentration. Enzyme activity drops above 10 mM of rac-BMBA (substrate inhibition). Activity plateau (represented by the dotted line) is then not reached, and enzyme is then not saturated by its substrate.

2 Loading

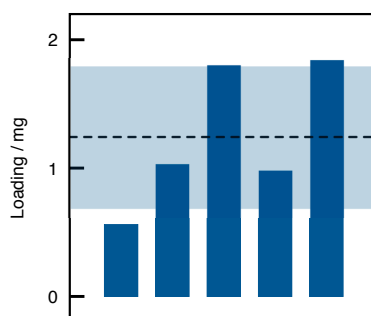


Figure A 4 – Loading determination. For enzyme immobilization, 50 mL of a glutaraldehyde buffered solution was injected through the flow reactor. Then, 50 mL of ATA-117 solution is injected (named “solution 0”). Typically, this enzymatic solution has a concentration of 0.2 mg/mL (called C_0). “Solution 1” was collected at the outflows (concentration C_1) and then homogenized. Finally, the reactor was washed with 50 mL of buffer (no enzyme) and 50 mL are collected at the outflows (solution 2, concentration C_2). As all the volumes were equal to 50 mL, the loading (L ; in mg) was determined by the following equation (mass balance): $L = 50 \text{ mL} \times (C_0 - C_1 - C_2)$. However, loading values were often low, and measurements suffered from high variability. Many experiments have been performed in order to identify the origin of that issue (data not shown): we ensured there was neither contamination nor interference involved in the loading determination. Temperature sensibility, reactants instability, and stirring method have also been studied but showed no influence on the measurement. Five repetitions of loading measurement have been achieved for this catalyst (TA-A50). Dotted line represents the mean value (1.2 mg); shaded area represents the relative standard deviation (RSD is 45 %).

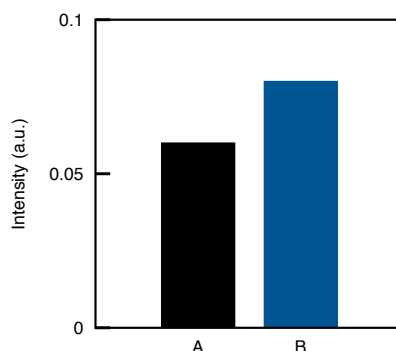


Figure A 5 – Dynamic light scattering of ATA-117 solutions. We observed that enzymatic solution concentration often increased by passing through the flow set-up, even without monolith. This suggests that initial enzyme solution (“solution 0”) may contain enzymatic aggregates that are not correctly probed by the Bradford titration method (C_0 is thus underestimated) and are broken only under flow constraints. Those aggregates in solution 0 are a source of variability. Dynamic light scattering (DLS) measurements confirm the aggregates hypothesis. Indeed, a higher intensity was observed around 3 nm for outflows enzyme solution, indicating that more free enzyme are available in the solution when passing through the flow set-up. Those free enzymes may come from the broken aggregates. The presence of aggregates in the “solution 0” causes high variability on loading measurement. Therefore, we are limited to a rough estimation on loading measurement. DLS signal intensity around 3 nm for ATA-117 solution (A) before and (B) after flow impregnation. Relative standard deviation is 15 %.

3 The adapted Bradford method

In the classical Bradford method, the protein concentration is directly linked to the absorbance of a solution at 595 nm [170]. Indeed, a blue colour appears when protein interacts with a dye, the Coomassie Brilliant blue (CBB).

However, in absence of protein, the CBB dye slightly absorbs at 595 nm (Figure A 6). Then, at low protein concentration, the relation between the absorbance at 595 nm and the protein concentration is not strictly linear.

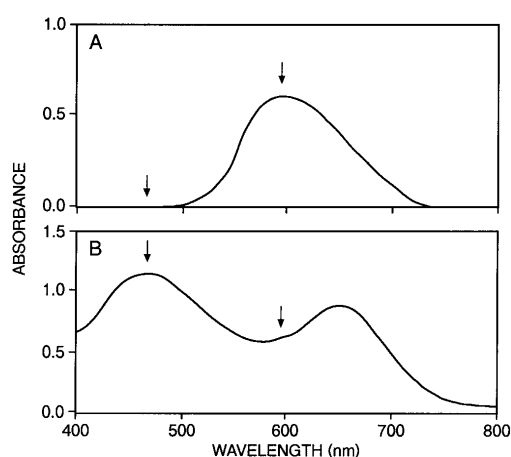


Figure A 6 – Spectra of the dye-protein complex (A) and the dye (CBB) alone (B). The arrows point at the absorbance maxima of 594 and 466 nm. Figure and caption taken from [171].

Zor *et al.* [171] proposed to take into account the others absorbance peaks (at 470 and 650 nm, related to the free CBB dye). Those two peaks intensity appears in the same proportions. The increase in the dye-protein complex concentration leads to a decrease in the free CBB concentration. Thus, an increase in the absorbance at 595 nm is inversely proportional to the decrease in the peaks at 470 nm, and then the absorbance ratio $\frac{A_{595}}{A_{470}}$ is linearly related to the protein concentration C_p :

$$\frac{A_{595}}{A_{470}} = m \cdot C_p + p$$

Line parameters m and p are determined by calibration curve. This modified Bradford method (in the calculation) lead to a more accurate assay for low enzyme concentrations.

4 Transaminases deactivation by NaBH_3CN

In order to check if the reducing agent could deactivate the transaminases, batch experiments were performed by adding reducing agent during the reaction (after one hour of reaction, Figure A 7, left). But, no deactivation occurred in batch reactors. Then, in a second batch experiment, the reducing agent was added to transaminases solutions prior to the batch reaction (one hour pre-incubation before the transamination reaction, Figure A 7, right). A strong deactivation was observed for transaminases solutions pre-incubated with NaBH_3CN .

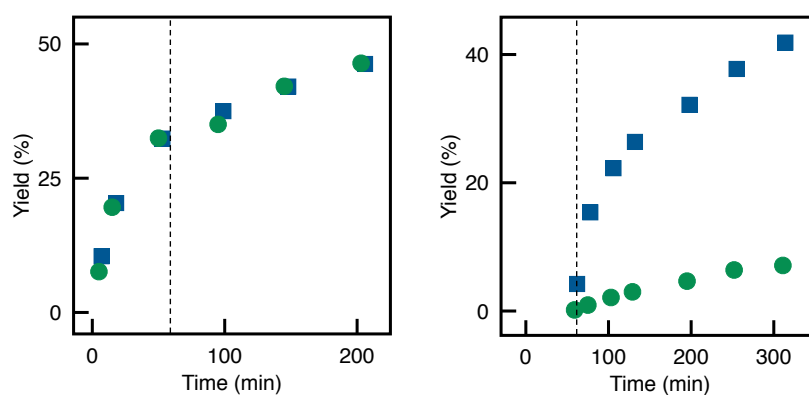


Figure A 7 – Effect of reductant on batch reactions. (Left) Effect of the addition of NaBH₃CN during the transamination in batch reactors (1h after starting the reaction, dotted line): (■) blank (no addition of NaBH₃CN); (●) addition of NaBH₃CN. Reactions conditions: 10 mM rac-BMBA, 10 mM pyruvate, 0.5 g/L PLP, 4.4 μM ATA-117, 5 % DMSO, NaBH₃CN 50 mM, 30 °C. (Right) Effect of pre-incubation with 50 mM NaBH₃CN on transamination reactions in batch reactors (1 hour pre-incubation, dotted line): (■) blank (no pre-incubation with NaBH₃CN); (●) one hour pre-incubation with NaBH₃CN. Reactions conditions: 10 mM rac-BMBA, 10 mM pyruvate, 0.5 g/L PLP, 4.4 μM ATA-117, 5 % DMSO, 30 °C.

5 Thermo-programmed calcination

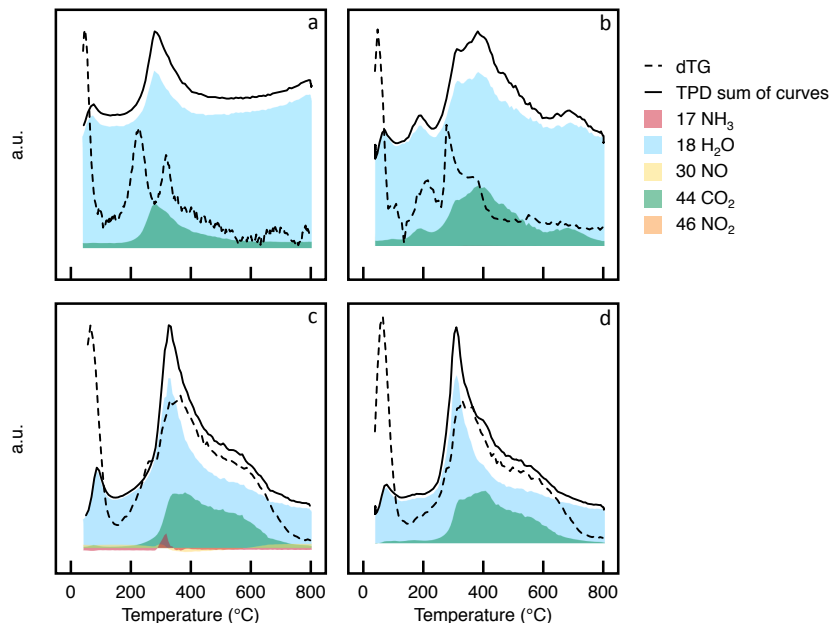


Figure A 8 – Thermo-programmed calcination for monoliths. (a) Si(HIPE), (b) M100_D, (c) A250_D and (d) M100A250_D. This allows identifying the nature of decomposed matter along with temperature. As expected, bare silica (a) releases only water and CO₂ (around 300 °C; due to organic contamination) when heated. Methyl-grafted monolith (b) also releases water and CO₂. However, the decomposition of organic matter occurs in a larger range of temperature (from 150 °C to 650 °C). APTES-grafted monolith (c) exhibit also water release and organic decomposition, but also ammonia releasing, due to the aminated function decomposition (around 300 °C). Hybrid hydrophobic and aminated monolith (d) exhibit similar organic decomposition (both carbon from MTES and APTES). Ammonia is not seen on this diagram (probably due to the so low amine concentration in the monolith).

6 Hypotheses on organic grafting structure and water concentration

Water desorbed on grafted samples was analysed by TGA and normalized by molar concentrations in MTES or APTES in order to see the impact of each mole of organic matter on the hydrophobicity (Figure A 9). We observed that MTES samples contained effectively less water than APTES samples. This is consistent with expected more hydrophobic properties of MTES samples.

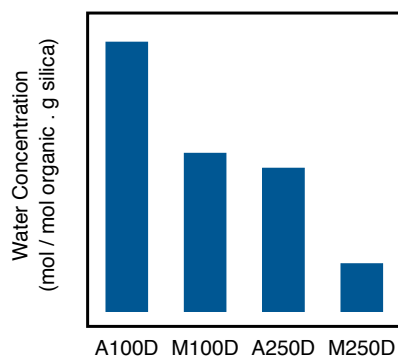


Figure A 9 – Water concentration, normalized by the molar organic concentration of each functionalized sample.

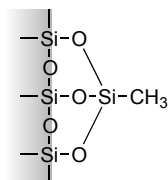


Figure A 10 – Expected structure of poly-dentate bounded MTES.

However, relative water amount seems to decrease with MTES concentration (whereas data are normalized by the molar concentration). Yet we expected that relative water amount remains constant with MTES concentration, as it is normalized by the MTES content. It would suggest that for the same MTES relative concentration, M250_D samples are more hydrophobic. This hypothesis is validated if M250_D exhibit MTES molecules bounded in a poly-dentate way (or due to lateral polymerization process; Figure A 10). Then, the hydrophobic moiety effectively grafted has a lower molar mass (40 g.mol⁻¹). It results that for the same mass grafted on the surface, more MTES molecules are grafted for M250_D samples than for M100_D samples, thus exhibiting a higher surface density of methyl groups. Under this hypothesis, the amount of water adsorbed on the monolith normalized by the molar content of organic is higher for M250_D (Figure A 11).

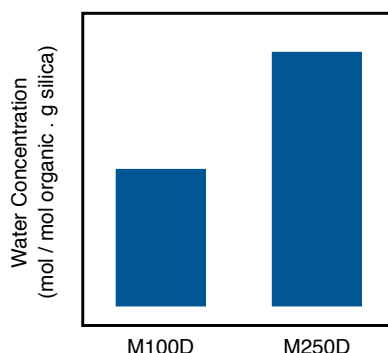


Figure A 11 – Water concentration, normalized by the expected molar organic concentration. M100_D and M250_D have undergone respectively a monodentate and a tridentate bounding.

7 Plasmid used for gene cloning

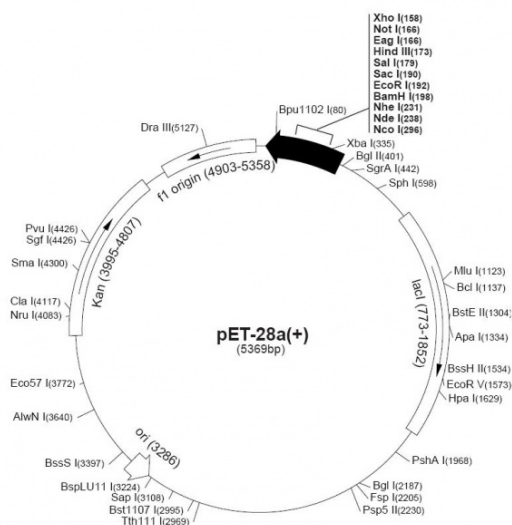


Figure A 12 – pET-28a(+) plasmid map. Image provided by Novagen.

His-tagged ATA-117 was produced by *E. coli* strain BL21(DE3) that is widely used as it allows very efficient transformations and the easy protein over-expression. His-tagged ATA-117 gene was inserted in a pET-28a(+) plasmid (Figure A 12). This plasmid allows the easy over-expression of inserted gene, using IPTG (isopropyl β -D-1-thiogalactopyranoside), and contains a kanamycin resistance gene (used for the screening).

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His-tagged ATA-117 gene (red-bold characters are the His-Tag; green-bod characters represent the trombone cleavage site):

MGSHHHHHSSGLVPRGSHMAFSADTSEIVYTHDTGLDYITYSDYELDPANPLAGG
AAWIEGAFVPPSEARISIFDQGYLHSDVTYTVFHVWNGNAFRLDDHIERLFSNAES
MRIIPPLTQDEVKEIALELVAKTELREAFVSVSITRGYSSTPGERDITKHPQVYM
YAVPYQWIVPFDRI RDGVHAMVAQSVRRTPRSSIDPQVKNFQWGD LIRAVQETHDR
GFEAPLLLDGDGLLAEGSGFNVVVIKDG VVRSPGRAALPGITRKT VLEIAESLGHE
AILADITLAELLDAD EVLGCTTAGGVWPFVSV DGNPISDGVPGPVTQSIIRRYWEL
NVESSSLTPVQY

8 Reaction conditions for Chapter IV

Table A 1 – Reaction conditions for flow reactions discussed in Chapter IV.

Biocatalyst	Enzyme concentration in impregnation solution (g/L)	Loading (mg)	Final yield (%)
His-TA-Si(HIPE)-30d	0.1	0.11	1.6
His-TA-G100 _B -NTA:Ni-30d	0.01	/	1.85
TA-G100 _B -30d	0.2	0.80	1.7
His-TA-G200 _B -NTA:Ni-30d	0.01	0.005	2
His-TA-G200 _B -NTA2h:Ni2h-30d	0.03	/	4.4
His-TA-G200 _B -NTA14h:Ni14h-30d	0.03	/	1.8
His-TA-G500 _B -NTA:Ni-30d	0.0281	0.006	2.5
TA-G500 _B -NTA:Ni-30d	0.0281	0.027	1.7
His-TA-G1000 _B -NTA:Ni-30d	0.03	/	2
His-TA-G1000 _B -NTA:Co-30d	0.03	0.21	Unstable
His-TA-GA-A250 _D -30d	0.0281	0.14	50
TA-GA-A250 _D -30d	0.2	0.13	16

9 Experimental conditions for mono and multi-enzymatic systems

Table A 2 – Experimental conditions for mono-enzymatic systems. Amino-donor and acceptor concentrations are expressed in mM, enzyme concentration in g/L (in brackets). For each reaction, PLP and DMSO concentrations were respectively 0.5 g/L and 5 %. PB and HEPES stand respectively for phosphate buffer and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer.

Figure	Amino-donor	Amino-acceptor	Buffer	Trans-aminase	Equilibrium displacement
Figure 5 - 3	XDD (10)	BAP (10)	PB pH 8	ATA-117 or ATA-256 (0.1)	Co-product polymerization
Figure 5 - 4, Left	XDD (110)	FPA (100)	HEPES pH 7.5	ATA-256 (1.5)	
Figure 5 - 6	ABA (100)	BAP (10)	PB pH 8	ATA-117 (0.5)	Co-product decomposition

Table A 3 – Experimental conditions for multi-enzymatic systems. Reactants concentrations are expressed in mM, enzyme concentration in g/L (in brackets). For each reaction, PLP and DMSO concentrations were respectively 0.5 g/L and 5 %.

Figure	Amino-donor	BAP	ATA-117	YADH	GDH	Glucose	NADH	NAD	Misc.
Figure 5 - 8, left	Isopropyl - amine (10)	10	0.1	0.02	0.02	10	0.1	0	
Figure 5 - 8, right	Isopropyl - amine (500)	10	2	1	1	50	1.5	0	
Figure 5 - 9	Isopropyl - amine (500)	10	2	1	1	50	1.5	0	85 μ L of H ₃ PO ₄ solution (85 % _w)
Figure 5 - 10, left	Isopropyl - amine (10)	10	2	1	1	50	0.1	0.1	

Annexes

Figure 5 - 10, right	Isopropyl - amine (500)	10	0.1	1	0.01 67	50	0.1	0.1	85 μ L of H_3PO_4 solution (85 % _w)
Figure 5 - 12	D- Alanine (50)	5	1	1	1	50	0.1	0.1	

10 Mono-enzymatic system using XDD and FPA

An attempt to catalyse the reaction between XDD and FPA was carried out with ATA-117. However, no conversion occurred, confirming that ATA-117 could not accept XDD as an amino-donor (Figure A 13).

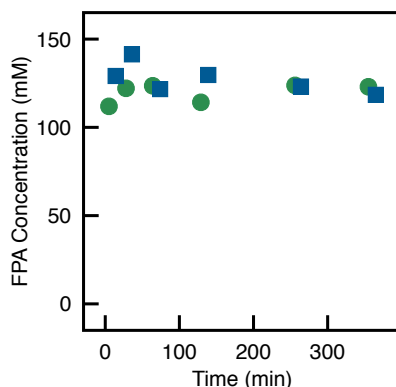


Figure A 13 – Reaction between XDD and FPA catalysed by ATA-117. Blue squares (■) represent the enzyme-catalysed reaction, green dots (●) is the blank test (no enzyme). Reaction conditions: XDD (110 mM), FPA (100 mM), PLP (0.5 g/L), ATA-117 (1.5 g/L), HEPES buffer pH 7.5. ATA-117 does not catalyse the reaction between XDD and FPA.

11 BAP solubility

BAP solutions were prepared by solubilising BAP into phosphate buffer (pH 8) containing 5 % DMSO. After the preparation (24 h), tiny precipitates were observed in the more concentrates vials. Supernatants were collected and analysed in gas chromatography (Figure A 14). BAP concentration in the supernatants reaches a plateau around 5 mM.

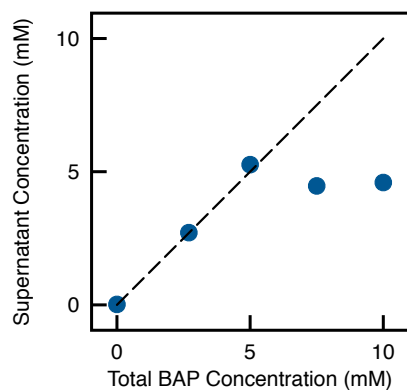


Figure A 14 – BAP solubility. BAP concentration prepared in the vials versus BAP concentration in the supernatant. BAP solubility is limited around 5 mM. Solutions were prepared in phosphate buffer (pH 8) containing 5 % DMSO.

12 NADH optimization for YADH

We suspected that high NADH concentration could inhibit the YADH. Then, as suggested by Cassimjee *et al* [225] we decide to decrease the NADH concentration and check out the activity of YADH (Figure A 15). Indeed, at low concentration (100 μ M), YADH activity is much higher than at high concentration. Specific activities are $9.27 \cdot 10^{-5} \text{ min}^{-1}$ (NADH 10 mM) and 0.0027 min^{-1} (NADH 100 μ M). Then, by adapting the NADH concentration to 100 μ M, global rate constant (k_{OBS}) is increased 29 times.

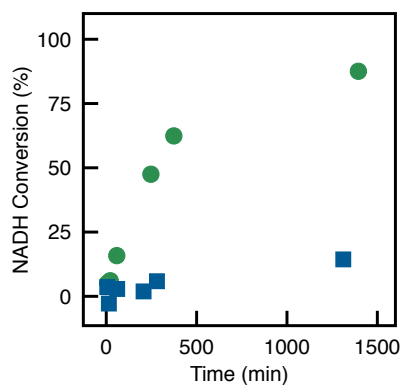


Figure A 15 – NADH conversion by YADH depending on initial NADH concentration. (■) 10 mM NADH ; (●) 100 μ M NADH.

13 Multi-enzymatic system using ethylamine as amino-donor

Using the multi-enzymatic system with ATA-117, YADH and GDH, we observed that YADH was the lowest enzyme. Then we wanted to enhance its activity. We suggested changing the substrate of YADH enzyme in order to have a more natural one, and then, it was expected to have a higher activity. In that purpose, we implement this multi-enzymatic cascade, using ethylamine as amino-donor (Figure A 16).

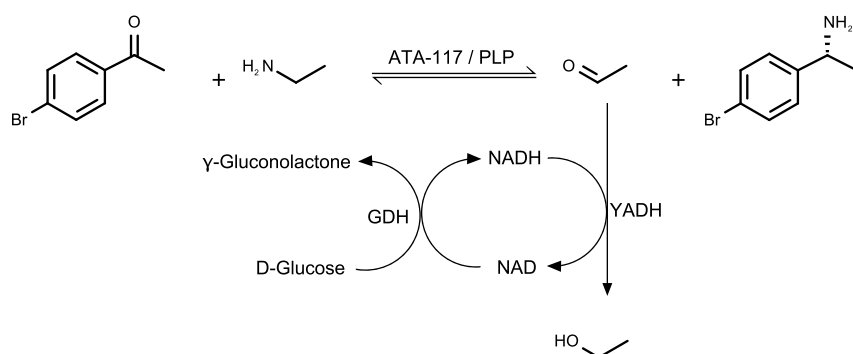


Figure A 16 – Multi-enzymatic method involving ATA, YADH and GDH. This reaction involves the BAP as amino-acceptor, and ethylamine as amino-donor. The ATA-117 catalysed reaction allows the production of acetaldehyde. This co-product is then transformed into ethanol by the YADH enzyme, using NADH as a cofactor. The NADH is recycled by the glucose oxidation reaction catalysed by the GDH enzyme.

The trial with ethylamine as amino-donor was conducted with high enzymes concentration (similar conditions as Figure 5 - 8, right; see Table A 3). Enzymatic cascade was active, but the BMBA production occurred slower than when isopropylamine was used as amino-donor (Figure A 17). A reaction catalysed by ATA-117 only was also conducted, and we observed a slight BMBA production over time. This suggests that, thermodynamically, the chemical equilibrium position is naturally quite favourable for the asymmetric synthesis. Indeed, the expected conversion (without equilibrium displacement) is 61 % (see Table A 6).

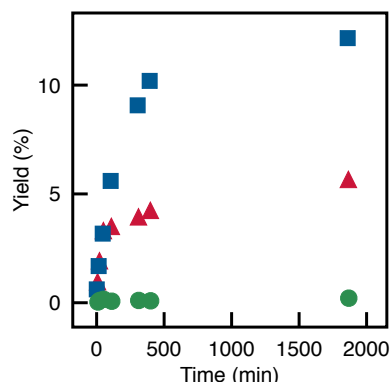


Figure A 17 – Effect of ethylamine as a substrate in the asymmetric synthesis. (■) Multi-enzymatic system using ethylamine as amino-donor. (▲) Asymmetric synthesis using only ATA-117. (●) Green dots represent the blank test (no enzyme).

We wondered if BAP could be converted in another product than BMBA by YADH or GDH. Indeed, those enzymes can catalyse the reduction of keto-compounds. In that way, BAP would be converted in bromo-phenylethanol (BPE) if reduced by any catalyst (Figure A 18). Then, we investigated if BPE was produced over time. BPE is quantified by gas chromatography. It results that GDH enzyme could slightly catalyse the conversion of BAP into BPE. However this BPE production remains low (less than 2 % after 1500 minutes of batch reaction) and would be neglected in flow mode (10 minutes contact time).

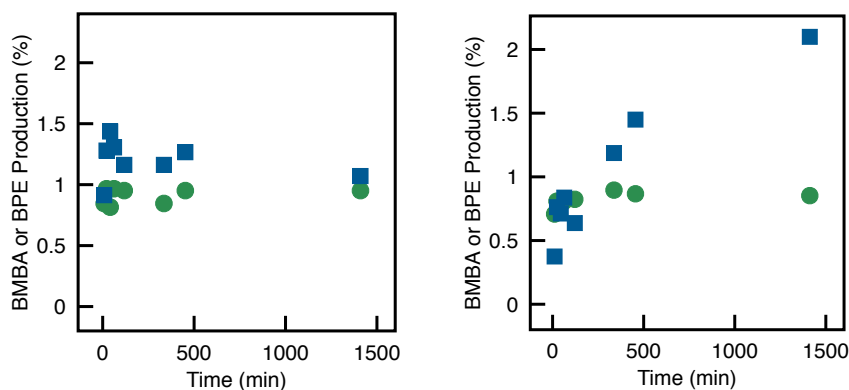


Figure A 18 – BAP conversion into BPE. Batch experiments were carried out to check if BAP could be converted into BPE by respectively YADH (left) or GDH (right) ((■) BPE production and (●) BMBA production). It exhibits that GDH enzyme could slightly catalyse the conversion of BAP into BPE. Reaction conditions were

Annexes

BAP (5 mM), NADH (100 μ M), NAD (100 μ M), enzyme (YADH or GDH 1 g/L), ethylamine (500 mM), PLP (0.5 g/L), glucose (50 mM).

14 Reaction conditions for flow asymmetric transaminations

Table A 4 – Reaction conditions for flow asymmetric transaminations.

Enzyme impregnation masses are expressed in mg ; BAP, BMBA, Pyruvate, Alanine and Glucose concentrations are expressed in mM ; NAD and NADH concentrations are expressed in μ M. Bed size is expressed in terms of number of monolith (of standard shape).

Figure	TA	YADH	GDH	BAP	BMBA	Alanine	Pyruvate	NADH	NAD	Glucose	Bed size
Figure 5 - 13	10	10	10	5	0	50	0	100	100	50	1
Figure 5 - 14 (left)	10	10	10	0	5	0	10	0	0	0	1
Figure 5 - 14 (mid.)	10	10	10	0	0	0	1	100	0	0	1
Figure 5 - 14 (right)	10	10	10	0	0	0	0	0	100	50	1
Figure 5 - 15	10	10	10	5	0	50	0	100	100	50	1
Figure 5 - 16	10	10	10	5	0	50	0	100	100	50	3
Figure 5 - 17	10	1*	1*	5	0	50	0	100	100	50	1
Figure 5 - 18	10 (ATA-117, His-TA or HEwT)	1*	1*	5	0	50	0	100	100	50	2

* YADH and GDH are continuously fed during the transamination. Values are expressed in concentration (in g/L) in the reaction medium.

Table A 5 – Yield and catalytic efficiencies of flow asymmetric transaminations. Flow rates are expressed in mL/min ; yields are expressed in % and catalytic efficiencies are in $\mu\text{mol}_{\text{BMBA}} \cdot \text{min}^{-1} \cdot \text{mg}_{\text{ATA}}^{-1}$ (see Introduction, Section 2.4.1 for calculations).

Figure	Flow rate	Yield	Catalytic efficiency
Figure 5 - 13	0,11	<1	0.00055
Figure 5 - 15	0,11	<0.5*	0.00028
Figure 5 - 16	0,11	<1	0.00055
Figure 5 - 17	0,11	~0	0
Figure 5 - 18	0,055	1.2 (ATA-117)	0.00033
		16 (His-TA)	0.0044
		2.5 (HEwT)	0.00069

* Yields were identical whether the impregnation sequence.

15 Chemical equilibrium position

For each reaction, equilibrium constant, K , has been calculated for reactions at 30 °C, using Gibbs free energy relations, where $\Delta_r G^\circ_T$ is the variation of standard free enthalpy associated to a reaction occurring at temperature T ; R is the gas constant.

$$K = \exp \left(-\frac{\Delta_r G^\circ_T}{R * T} \right)$$

The term $\Delta_r G^\circ_T$ can be calculated according to this relation, where $\Delta_r H^\circ_T$ is the standard enthalpy associated to the reaction, and $\Delta_r S^\circ_T$ is the standard entropy associated to the reaction.

$$\Delta_r G^\circ_T = \Delta_r H^\circ_T - T \cdot \Delta_r S^\circ_T$$

Standard enthalpy and entropy associated to the reactions can be calculated with the following; where $\Delta_r H^\circ_{298}$ is the standard enthalpy of the reaction occurring at 298 K; $\Delta_r C_p$ is the variation of heat capacity at constant pressure between reactants and products and $\Delta_r S^\circ_{298}$ is the standard entropy associated to this reaction at 298 K.

$$\Delta_r H^\circ_T = \Delta_r H^\circ_{298} + \int_{298}^T \Delta_r C_p dT$$

$$\Delta_r S^\circ_T = \Delta_r S^\circ_{298} + \int_{298}^T \frac{\Delta_r C_P}{T} dT$$

The term $\Delta_r C_P$ is calculated according to the stoichiometric coefficient and the heat capacity of each compound, using the following relation, where v_i is the stoichiometric coefficient (negative for reactants):

$$\Delta_r C_P = \sum v_i \cdot C_{Pi}$$

The terms $\Delta_r H^\circ_T$ and $\Delta_r S^\circ_T$ are calculated using the following relations, where $\Delta_f H^\circ_{i,298}$ and $S^\circ_{i,298}$ are respectively the standard formation enthalpy of the compound i , and the standard entropy of the compound i at 298 K.

$$\Delta_r H^\circ_{298} = \sum v_i \cdot \Delta_f H^\circ_{i,298}$$

$$\Delta_r S^\circ_{298} = \sum v_i \cdot S^\circ_{i,298}$$

Heat of formation, heat capacity at constant pressure and entropy of each compound at 298 K has been computed using *molcalc.org* tool [226]. Then, from the equilibrium constant and the initial reactants concentration, it was possible to predict the expected conversion for asymmetric synthesis (without any equilibrium displacement; Table A 6).

Table A 6 – Chemical equilibrium position for the transamination reactions studied. Initial reactants concentrations are expressed in brackets (in mM). Expected conversion is the conversion of BAP at the equilibrium. BAP, XDD, BMBA, FBA, ABA, AAA, IPA, EtAm, AP and MBA, respectively stand for 4-bromo-acetophenone, o-xylylenediamine, 4-bromo-1-methylbenzylamine, 2-formylbenzylamine, 3-DL-aminobutyric acid, acetoacetic acid, isopropylamine ethylamine, acetophenone and methylbenzylamine.

Transamination reaction	Scheme	K	Expected conversion
BAP (10) + XDD (10) → BMBA + FBA	Figure 5 - 2	2.95	63.22 %
BAP (10) + ABA (100) → BMBA + AAA	Figure 5 - 5	3.30	96.85 %
BAP (5) + IPA (500) → BMBA + Acetone	Figure 5 - 7	0.00028	11.15 %

BAP (5) + EtAm (500) → BMBA + Acetaldehyde	Figure A 17	0.0098	61.35 %
BAP (5) + Alanine (50) → BMBA + Pyruvic acid	Figure 5 - 11	$1.48 \cdot 10^{-8}$	0.04 %
AP (5) + Alanine (50) → MBA + pyruvic acid	/	$6.28 \cdot 10^{-6}$	0.79 %

From those data, it is observed that when using brominated substrates (BAP), the chemical equilibrium is less favourable to the ketone amination. In the absence of brome (e.g. acetophenone – AP), the equilibrium constant is higher and then, the AP amination is more favoured. Indeed, brome atoms have positive mesomeric properties, and thus help to stabilize the carbonyl bound in the keto compound. Thus, the amination of that carbonyl function is less favourable. Using negative mesomeric functions (e.g. NO₂ or CN) instead of Br (in *para* position) would lead to increase the equilibrium constant, and thus, would favour the amination of keto substrate.

16 *Fj*TAL protein sequence

MGSSHHHHHSSGLVPRGSHMASMTGGQQMGRDGMNTINEYLSLEEFEAIIIFG
 NQKVTISDVVVNRVNESFNFLKEFSGNKVIYGVNTGFGPMAQYRIKESDQIQLOYN
 LIRSHSSGTGKPLSPVCAKAAAILARLNTLSLGN SGVHPSVINLMSELINKDITPLI
 FEHGGVGASGDLVQLSHLALVLIGEGEVFYKGERRPTPEVFEIEGLKPIQVEIREG
 LALINGTSVMTGIGVVNVYHAKLLDWSLKSSCAINELVQAYDDHFS AELNQT KRH
 KGQQEIALKMRQNLS DSTLIRKREDHLYSGENTEEIFKEKVQEYYS LRCVPQILGP
 VLETINNVASILEDEFNSANDNPIIDVKNQHVYHGGNFHGDYISLEMDK LKIVITK
 LTMLAERQLNYLLNSKINELLPPFVNLGTLGFNFGMQGVQFTATSTTAE SQMLSNP
 MYVHSIPNNNDNQDIVSMGTNSAVITSKVIENAFEVLAIEMITIVQAIDYLGQKDK
 ISSVSKKWYDEIRNIIPTFKEDQVMYPFVQKVKDHLINN

