Challenges of Chemoenzymatic and Biocatalytic Reaction Cascades and Online Monitoring at a Microscale

A thesis submitted to the Department of Biochemical Engineering University College London (UCL) for the degree of Doctor of Philosophy by

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2019
Declaration

I, Pia Gruber, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. The thesis does not exceed the prescribed word limit and none of the work presented in this thesis has been submitted for a qualification at any other institution.

Signature:                     Date:       March 2019, London, UK
Acknowledgement

I want to thank my friends and family for supporting me.

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All ethics issues relevant to this work have been considered and approval is not required.
Abstract

Microreactors have been established as useful platforms for chemical and biocatalytic reaction cascades. These are often difficult to implement due to substrate, product, solvent, cofactor, or pH-based inhibitions or unfavourable reaction equilibria, but the small dimensions and physical advantages of microreactors allow a cost-effective optimisation of such reactions. The implementation of sensors adds insight into the reaction progress and eases the reaction optimisation process.

In this work, reactor designs, sensor integration protocols and reaction optimisation guidelines have been developed for two types of reaction cascades, namely chemoenzymatic and bi-enzymatic cascades. The chemoenzymatic reaction couples a Diels-Alder reaction with transketolase to produce 1-(3′,4′-dimethylcyclohex-3′-enyl)-1,3-dihydroxypropan-2-one (DCDHP) and the two-enzyme cascade uses transketolase and transaminase to produce 2-amino-1,3,4-butanetriol. Optimisation of reaction conditions led to a 10 mM 2-amino-1,3,4-butanetriol production in two hours. The implementation of an aluminium chloride packed-bed reactor and use of acetonitrile as a solvent for the Diels-Alder reaction led to the production of 200 mM intermediate in only 50 min and a total process yield of 3.5 mM DCDHP in the coupled Diels-Alder – transketolase reaction after only 200 min. Sensors suitable for monitoring a range of pH 3.5-8.5 were developed to monitor reactions in which pH shifts occur due to product formation. Additionally, a carbon dioxide sensing system has been adapted for implementation into a microfluidic reactor to monitor the transketolase reactions used in both model systems, in which the side-product formation of carbon dioxide is used to drive the reaction. Finally, pH sensors were implemented into a microfluidic side-entry reactor to record a real-time pH profile at eight different locations in the reactor. This made manual adjustment of the pH in the reactor possible and resulted in a higher reaction yield. This shows that online monitoring can be used to improve reaction yields for enzymatic reactions at a microfluidic scale.
Impact statement

The guidelines that were developed in order to successfully achieve conversion in the reaction cascades shown in this thesis are universally applicable for both chemo-enzymatic and biocatalytic reaction cascades. These cascades, presented in Chapter 2 and Chapter 3, selectively yield chiral products relevant for the production of pharmaceutical precursors from low-cost achiral substrates. The reactions were performed in microreactor cascade setups with the aim to produce chiral precursor molecules that can be used for the synthesis of antibiotics, brain lipids and glycosidase inhibitors. They are therefore of interest to the pharmaceutical industry. The microreactor-scale setups and the enzymatic reaction steps replace complex multistep chemical syntheses, which would generally require intermediate purification steps and result in the waste of large quantities of organic solvents. This makes the presented reactions more economical and eco-friendly.

Additionally, analytical protocols, procedures and sensor technology have been developed in order to enable substrate and product quantification and reaction monitoring for the presented reaction cascades. These can be readily applied to aid future projects. The advances in sensor technology, developed in cooperation with Graz University of Technology (TUG) and presented in Chapter 4 and 5, can be used for the monitoring of pH and carbon dioxide at a microfluidic scale. The pH sensor can be used for the real-time monitoring of reaction progress and the carbon dioxide sensors can be used for the closing of mass balances for biocatalytic reactions.

The microfluidic side-entry reactor, as presented in Chapter 5, could be used for the rapid determination of a reaction’s pH profile and for faster optimisation of reaction conditions based on the adjustment of this profile. This process could be automated in a future project.

The work presented in this thesis was published in four peer-reviewed scientific journals and presented at five international conferences in the form of two talks and three posters. The publication and presentation details can be found listed in the appendix (Appendix 8.1) and will be referred to in the thesis text where relevant.
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Nomenclature

Symbols

A1 dphi value at low fluorescence
A2 dphi value at high fluorescence
dx Slope at point of inflection
hυ Photon emission
τ Residence time [min]
σ Enzyme activity
S0 Unexcited singlet state
S1/S2 Excited singlet state
t Reaction time
T1 Excited triplet state
x₀ Point of inflection

Abbreviations

6-APA 6-amino benzyl penicillanic acid
ABT 2-amino-1,3,4-butanetriol
AchE Acetylcholine esterase
AP Acetophenone
BSA Bovine serum albumin
CALB Candida antarctica lipase B
CCA 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde
CCD Charge-coupled device
CNC Computerised numerical control
CO₂ Carbon dioxide
DA Diels-Alder reaction
DCDHP 1-(3',4'-dimethylcyclohex-3'-enyl)-1,3-dihydroxypropan-2-one
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>DLR</td>
<td>Dual Lifetime Referencing</td>
</tr>
<tr>
<td>dphi</td>
<td>Change in phase angle (phi)</td>
</tr>
<tr>
<td>E(t)</td>
<td>Total amount of enzyme</td>
</tr>
<tr>
<td>E.coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ee</td>
<td>Enantiomeric excess</td>
</tr>
<tr>
<td>ERY</td>
<td>L-erythrulose</td>
</tr>
<tr>
<td>F</td>
<td>Flow rate</td>
</tr>
<tr>
<td>FID</td>
<td>Flame ionization detector</td>
</tr>
<tr>
<td>GA</td>
<td>Glycolaldehyde</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>GOx</td>
<td>Glucose oxidase</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HF</td>
<td>Hydrogen fluoride</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HPA</td>
<td>Lithium–β-hydroxypyruvate</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HPTS</td>
<td>8-Hydroxypyrene-1,3,6-trisulfonic acid</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IC</td>
<td>Internal conversion</td>
</tr>
<tr>
<td>ID</td>
<td>Inner diameter</td>
</tr>
<tr>
<td>IEMR</td>
<td>Immobilised enzyme microreactor</td>
</tr>
<tr>
<td>IPA</td>
<td>Isopropylamine</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>ISC</td>
<td>Intersystem crossing</td>
</tr>
<tr>
<td>ISFET</td>
<td>Ion-sensitive field effect transistor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>ISPR</td>
<td><em>in-situ</em> product removal</td>
</tr>
<tr>
<td>ISSS</td>
<td><em>in-situ</em> substrate supply</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LED</td>
<td>Light-emitting diode</td>
</tr>
<tr>
<td>MBA</td>
<td>(S)-α-Methylbenzylamine</td>
</tr>
<tr>
<td>MBR</td>
<td>Microbioreactor</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometer</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>Nickel nitrilotriacetic acid</td>
</tr>
<tr>
<td>NIR</td>
<td>Near infrared</td>
</tr>
<tr>
<td>NRT</td>
<td>Normalised residence time</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PEN</td>
<td>Polyethylene naphthalate</td>
</tr>
<tr>
<td>PET</td>
<td>Polyethylene terephthalate</td>
</tr>
<tr>
<td>PFA</td>
<td>Perfluoroalkoxyalkane</td>
</tr>
<tr>
<td>PG</td>
<td>Penicillin G</td>
</tr>
<tr>
<td>PGA</td>
<td>Penicillin G acylase</td>
</tr>
<tr>
<td>PLP</td>
<td>Pyridoxal-5-phosphate monohydrate</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>rISC</td>
<td>Reversed intersystem crossing</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RTD</td>
<td>Residence time distribution</td>
</tr>
<tr>
<td>SHM</td>
<td>Staggered Herringbone Mixer</td>
</tr>
<tr>
<td>TAm</td>
<td>Transaminase</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ThDP</td>
<td>Thiamine pyrophosphate</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>THN</td>
<td>1,3,6,8-tetrahydroxynaphthalene</td>
</tr>
<tr>
<td>TK</td>
<td>Transketolase</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TUG</td>
<td>Technische Universität Graz</td>
</tr>
<tr>
<td>UCL</td>
<td>University College London</td>
</tr>
<tr>
<td>U</td>
<td>Unit for enzyme activity</td>
</tr>
<tr>
<td>UDP-galactose</td>
<td>Uridine diphosphate galactose</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>UMP-galactose</td>
<td>Uridine monophosphate galactose</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V_s</td>
<td>Reaction volume</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>µSER</td>
<td>Microfluidic side-entry reactor</td>
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1. Introduction

Chemical processes have been at the core of pharmaceutical production for much of the previous centuries (Pollard and Woodley, 2007). A major drawback of purely chemical synthesis is the inherent difficulty of producing chiral molecules selectively (Pollard and Woodley, 2007). The importance of enantiomeric selectivity has been shown in the effect of Thalidomide, where the use of enantiomeric mixtures led to the considerable deformation of limbs in infants born to mothers who were treated with the drug for headaches (Smithells, 1965).

Separating enantiomeric mixtures can pose a challenge, both at bench- and industrial-scale productions. Therefore, it would be preferable to be able to produce just the desired enantiomers in the first place. Biocatalysis can be a solution to this challenge. Enzymatic reactions are usually enantioselective, either by only accepting one enantiomer as a substrate, or by producing only one of the possible enantiomers (Wang and Reetz, 2015). In order to serve as a viable alternative to chemical synthesis, biocatalytic reactions have to be able to show a clear advantage by achieving comparable or improved yields, reducing reaction time, being more cost-effective or performing the reaction more selectively (Wang and Reetz, 2015, Pollard and Woodley, 2007, Rudroff et al., 2018).

In addition to the increased utilisation of biocatalysis in pharmaceutical productions, there is a growing interest in the pharmaceutical industry to shift productions from batch-mode to continuous operations (Plumb, 2005). Continuous processes have already been shown to be useful alternatives to batch processes when it comes to chemical reactions. This is due to the possibility of cost-effective process development, safer production processes (when combined with miniaturisation) with emphasis on toxic or corrosive substrates and a potential for higher conversions. Continuous production can reduce reactor down-time for cleaning procedures.
Among the advantages are a consistent product quality output rather than individual batch qualities, a potential to avoid side reactions once reaction conditions have been optimised, a wider possible temperature range due to enhanced process safety in smaller volumes and a reduction of reaction time and solvent use (Costantini et al., 2013, Jähnisch et al., 2004). Reactors with sub-millimetre to sub-micrometre dimensions (Wohlgemuth et al., 2015), have already found applications in solid-liquid, liquid-liquid and gas-liquid reactions, photochemistry, radiochemistry and the manufacturing of nanoparticles (Whitesides, 2006, Mark et al., 2010, Jähnisch et al., 2004, Yoshida, 2008, Wiles and Watts, 2010, Porta et al., 2015). For chemical cascades, the use of microfluidic volumes is especially useful for reactions that require the use of explosive, toxic, or otherwise hazardous components (Singh et al., 2016). Microfluidic reactor setups have been successfully used in single and multi-step organic synthesis (Pfeiffer and Nagl, 2015, Rackus et al., 2015, Webb and Jamison, 2010, Bana et al., 2016, Porta et al., 2015). A further advantage of microfluidic cascades over conventional multistep synthesis involving several steps is the avoidance of intermediate extraction and purification. Often, the traditional ‘round-bottomed flask approach’ requires several additional steps from protection, deprotection, extraction and purification before the next synthesis step can occur. Each additional step in a synthesis process usually means a reduction of overall yield. This can be avoided by using a well-optimised continuous microfluidic flow system.

When it comes to the production of pharmaceutically relevant compounds, such as chiral building blocks, microfluidic systems offer an attractive alternative to bench-scale production processes, especially when they are used as vessels for performing complex, cascading biocatalytic reactions. These reactions can be used for the production of chiral products, especially when the synthesis leads to unstable intermediates, as the small fluid volumes allow swift fluid handling and make subsequent reactions fast enough to allow a cascading reaction to continue without requiring intermediate purification or isolation. The choice of materials and channel dimensions suitable for the application allows the reactions to be carried out on a much
smaller scale, making screenings for ideal conditions faster and more cost-effective, as no large quantities of chemicals are required. However, cascading reactions are challenging since each enzyme in the cascade has its own optimal reaction conditions, such as temperature and pH. Furthermore, substrates or products of one enzyme can act as inhibitors of other enzymes or even cause unwanted side-reactions (Chen et al., 2006). It is therefore necessary to develop guidelines for the optimisation of such reaction systems in order to maximise yield, while also minimising inhibition and side reactions. Once guidelines are established, the value of microreactors for biocatalytic process design can be better assessed.

The area of research referred to as ‘microfluidics’ developed from the advances made in technology for electronics manufacturing in the mid-1980s. During this time, the production of micro-valves and micro-pumps was made possible due to miniaturisation techniques developed for the fabrication of microelectronic components (Nguyen, 2002).

Many definitions are in use regarding the term ‘microfluidic system’. According to Nguyen (Nguyen, 2002), a setup can be characterised as ‘microfluidic’ if the fluid processing area, such as the reaction channel or chamber, is miniaturised to sub-millimetre size. In his seminal review on the origin and future of microfluidics, Whitesides defines microfluidics as ‘the science and technology of systems that process or manipulate small (10⁻⁹ to 10⁻¹⁸ litres) amounts of fluids, using channels with dimensions of tens to hundreds of micrometres’ (Whitesides, 2006).

Microfluidics has been applied as a useful tool in multiple fields; among them are clinical applications, forensic science, bioprocess engineering, biocatalysis, environmental monitoring and pharmacology (Bruus, 2008). Flow reactions in structured microfluidic reactors provide promising methodologies and have attracted a broad interest for biocatalytic process design. This interest has arisen from several advantages associated with the use of microfluidic reactors, such as: increased control over reaction conditions (such as time, pH, flow rates and reagent addition), reduced use of resources, increased mass and heat transport due to the high surface-to-volume ratio, short diffusion paths and the option of compartmentalisation of multi-enzyme
reaction. Furthermore, energy consumption is reduced, process integration is possible (O'Sullivan et al., 2012) and increased throughput can be achieved via parallelisation (Bolivar et al., 2011; Krühne et al., 2014) (Wohlgemuth et al., 2015). Flow chemistry is frequently used to find novel routes for process intensification and improved process economics (Bolivar et al., 2011). These advantages may potentially contribute to overcoming the time constraints associated with biocatalytic process development, along with the reduction of costs and environmental impact.

The same advantages, already well documented for chemical cascades, potentially apply to chemoenzymatic and multi-enzyme reactions in microreactors (Bolivar et al., 2011; Wohlgemuth et al., 2015), though not all have been demonstrated yet. In the following section, successful cascading microreactor systems will be reviewed. For this purpose, spatially separated reactions, meaning each reaction step is performed in a separate reactor while using the product of the previous reactor as a substrate for the subsequent reaction, will be summarised. Spatial process confinement can ease the individual optimisation of each reaction and decrease the cross-reactivity of reaction components, though this is not a guarantee against inhibitory effects and side reactions, which need to be addressed during the optimisation process (Chen et al., 2006, Gruber et al., 2017b). Furthermore, compartmentalisation enables the integration of sensor technology into the corresponding reaction step that requires monitoring. Microfluidic chemoenzymatic or enzymatic reaction cascades could therefore be used as a cost-effective, versatile tool to optimise the syntheses of new enantiomeric molecules.

In order to give an overview of previous successful cascade reactions, the first part of this chapter will focus on the development of chemo-enzymatic and enzyme-enzyme microfluidic reaction cascades. The second part of this chapter will focus on the fabrication of the microfluidic reactors and the third part will focus on implementation of online monitoring approaches for the monitoring of process variables for enzymatic reactions.
1.1. Coupled Chemo-Enzymatic Reactions

While a large variety of multi-step chemical reactions have been realised in continuous flow microreactors (Porta et al., 2015), only very few chemo-enzymatic reaction cascades have been reported. These reactions, which consist of a chemical reaction followed by an enzymatic one (or vice versa), are especially interesting for the development of new synthetic routes for chiral compounds, particularly for the reduction of the number of synthesis steps.

The main challenge for chemo-enzymatic cascades lies in the matching of reagents. While the chemical step usually requires organic solvents, either a single solvent or a mixture of solvents, the enzymatic step requires aqueous conditions. Enzymes usually show a reduced activity when in contact with an organic solvent. It is therefore necessary to match the two reaction media in such a way that a suitable enzyme activity is achieved and that little to no inhibition or deactivation of the enzyme takes place. If the presence of the organic solvent causes denaturation of the enzyme, clogging can occur in the channel, which needs to be avoided. Therefore, the enzymes should be tested for long-term operation stability.

Multi-phase reactors for biocatalytic reactions have been reviewed by Žnidaršič-Plazl (Žnidaršič-Plazl, 2014, Žnidaršič-Plazl, 2017) and Karande et al. (Karande et al., 2016). One of few examples for a chemo-enzymatic cascade is the synthesis of 2-aminophenoxyazin-3-one using three separate microfluidic devices and achieving 18.9% yield (Luckarift et al., 2007) (Table 1). The reactors, in order, contained metallic zinc, silica-immobilised hydroxyaminobenzene mutase and silica-immobilised peroxidase. Another example was presented by Delville et al. (Delville et al., 2015) who used an (R)-selective hydroxynitrile lyase to synthesise the stereoisomers of various unstable cyanohydrins from aldehydes. To prevent racemisation, a second reaction step was added to add a protecting group to the cyanohydrin. The compartmentalisation of the reactions allowed the combination of incompatible reaction conditions through an in-line separator module. Due to this, yields of
68% and 97% enantiomeric excess (ee) were achieved and one work-up and extraction step was avoided, thereby shortening the process.

A way to minimise sensitivity to solvent content in the aqueous phase might be directed enzyme evolution or protein design (Fornera et al., 2012), since most important parameters for a successful chemo-enzymatic cascade relate to enzyme activity, stability, and how these properties are influenced by organic solvents, catalysts, substrates, by-products, pH and temperature (Dalby, 2011). Enzyme engineering could improve solvent resistance and enhance the enzyme’s activity towards conversion of non-native substrates (Dalby, 2003, Reetz, 2016).

1.2. Coupled Enzyme-Enzyme Reactions

While inhibition and inactivation through chemical components and solvents is less likely to occur in a multi-step enzyme cascade, compared with a chemo-enzymatic reaction cascade, there are still challenges to overcome in order to achieve a successful enzymatic reaction. This includes the matching of reaction conditions, flow rates, activities, buffers, pH and temperature, as well as the overcoming of inhibition effects through unreacted substrate and cofactors (Ardao et al., 2013). Here, again, the compartmentalisation of the reactions can help to optimise the reaction conditions for each biocatalyst and prevent cross-inhibition. Figure 1 illustrates all potential sources of inhibition that can arise in a theoretical enzymatic cascade.
In a case such as this example, where the intermediate, cofactor and product are prone to degradation and causing inhibition, an intermediate step which removes inhibiting reactants or products might be necessary to achieve high conversion. In order to prevent side reactions with substrates of preceding reaction steps, the removal or retention of the enzyme might be necessary, for example using an in-line filtration removal step (O'Sullivan et al., 2012), or the use of immobilised enzymes, could be considered. This could also allow re-cycling and re-use of the enzyme (Figure 2). In situ substrate supply might be necessary to overcome the inhibition effects of the substrates of any step.
In order to retain enzymes in a reactor, the enzymes are usually attached to the surface of carriers such as beads through various immobilisation techniques, or entrapped in porous matrices (Matosevic et al., 2011b, Bajić et al., 2016, Miložič et al., 2017, Carvalho et al., 2017, Carvalho and Fernandes, 2015, Abdul Halim et al., 2013, Carvalho et al., 2016, Bolivar et al., 2017, Valikhani et al., 2016). The amount of enzyme that can be immobilised in any empty or packed channel is finite and limited by the amount of accessible surface area and reactor volume. The use of free enzyme instead of immobilised enzyme could allow a higher enzyme activity per unit area within the channel because the enzyme can move within the whole reactor volume.

While the use of free enzyme can be used to increase the reaction speed, immobilisation techniques can lead to a longer stability, therefore the choice between applying free or immobilised enzyme must be made according to the reaction system’s requirements. For the retention of free enzymes in each compartmentalised reactor, filtration devices as reported by O’Sullivan et al. (O’Sullivan et al., 2012) could be used. A major advantage of multi-enzyme reaction cascades is the possibility of mimicking in vitro biosynthetic reactions (Lee et al., 2003). Using the modular approach of compartmentalised
reactors, it is possible to perform metabolic reaction cascades that are performed by cells or organs in nature, or create de novo pathways to produce new molecules. Being able to evaluate the effects of new reaction conditions or enzyme variants provides a strong basis for developing new multi-step biosynthetic reactions. A number of two- and three-step enzyme cascades have been reported in literature, though only a limited number of them are used for synthesis of organic compounds (Table 1). Ku et al. (Ku et al., 2006) created a synthetic pathway consisting of a type III polyketide synthase, 1,3,6,8-tetrahydroxynaphthalene (THN) synthase and a soybean peroxidase, all immobilised in an enzymatic microreactor. THN synthase was immobilised on prepacked Ni–NTA agarose beads in a microfluidic channel and the soybean peroxidase was covalently attached to the channel walls of a second reactor, which had been pre-coated with a reactive poly (maleic anhydride) derivative. Lee et al. (Lee et al., 2003) produced novel polyketide derivatives such as flaviolin and biflaviolin at a yield of 40% using immobilised enzymes on a glass surface. Babich et al. (Babich et al., 2012) demonstrated the production of a complex chiral carbohydrate analogue in a three-step cascade reaction with acid phosphatase, fructose-1,6-diphosphatase aldolase and acid phosphatase in a cascade of packed bed reactor. Liu et al. (Liu et al., 2002) presented a seven step reaction system to produce uridine diphosphate galactose (UDP-galactose) in which all enzymes were immobilised on Ni-NTA agarose beads that were packed into a column.
An immobilised enzyme microreactor (IEMR) was developed for the characterisation of a de novo transketolase-ω-transaminase cascade to synthesise chiral amino alcohols (Matosevic et al., 2011a). For this purpose, two surface-derivatised silica capillaries were used to immobilise the two His₆-tagged enzymes. In the first reaction step, a transketolase is used to produce a chiral ketone from achiral substrates and in the second reaction transaminase catalyses the ketone to a chiral amino alcohol.

### 1.3. Cascades as Tools for Online Monitoring

The monitoring of enzymatic reactions, as individual or cascades, provides insight into the reaction conditions and can significantly reduce the process development time and enhance insight into the reaction progress. A number of optical (Pfeiffer and Nagl, 2015, Sun et al., 2015), optofluidic (Yue et al., 2012, Chrimes et al., 2013), spectroscopic and electrochemical (Rackus et al., 2015) detection methods are available for on-line and real-time measurements for process variables such as oxygen, pH, glucose, carbon dioxide, or specific reactants. So far, these methods have been used for the monitoring of few individual enzymatic reactions and
even more rarely for microfluidic enzymatic cascades. (Gruber et al., 2017d, Ehgartner et al., 2016a, Ehgartner et al., 2016b, Ungerböck et al., 2013b, Pfeiffer et al., 2017). One explanation for this is that the detection methods depend on specific chemical, spectroscopic, or optical properties; therefore, a generalised monitoring approach suitable for multiple systems is rarely achievable. Development or optimisation of sensing principles is usually required in order to develop bespoke online monitoring techniques to a given reaction.

In cases where sensor technology has been used to monitor cascading systems, the monitoring aspect was designed to indirectly quantify the reaction using a side reaction or side-product (Costantini et al., 2013, Wei et al., 2016). This approach is possible when the cascade reaction transforms an otherwise not easily quantified analyte into a chemical species that is more easily monitored, for example through the use of oxygen sensors. A common cascade model system is the coupling of glucose oxidase (GOx) with horseradish peroxidase (HRP) in order to monitor the oxygen changes in the system. That is accomplished through a proportionate colour-change, caused by the production of hydrogen peroxide in the presence of the oxygen and glucose (Costantini et al., 2013, Lin et al., 2014). This kind of detection system has been used by Wei et al. (Wei et al., 2016), where this cascade of enzymes is used to detect cocaine in urine samples, providing a point of care device that is equipment-free and based on capillary action only. A variant of this system was presented by Fornera et al. (Fornera et al., 2012) where β-galactosidase was added to the GOx-HRP couple to measure lactose. Another three-enzyme cascade in microreactors and capillaries was achieved by Vong et al. (Vong et al., 2011) who coupled Candida antarctica lipase B (CALB) to the GOx-HRP couple. The CALB and GOx were immobilised in the reactors using a novel ssDNA-ssDNA interaction method to immobilise the enzymes in discrete zones. Product increase was monitored using absorbance measurements through the capillary walls.

Atalay et al. (Atalay et al., 2009) presented enzyme cascades to differentiate and quantify sucrose, D-glucose and D-fructose in one assay using an array of enzymes (β-fructosidase,
hexokinase, glucose-6-phosphate dehydrogenase, phospho-glucose isomerase). Ferrer et al. (Ferrer et al., 2014) developed a “paper microfluidic-based enzyme catalysed double microreactor assay” using the enzymes lactate dehydrogenase and diaphorase to create a fluorescent complex that could be detected through optical readout. A cascade of acetylcholine esterase (AchE) and choline oxidase has been used to detect organophosphorus compounds by Han et al. (Han et al., 2012).

Logan et al. (Logan et al., 2007) patterned invertase, horseradish peroxidase and glucose oxidase onto porous polymer monoliths in separate regions of a single channel. Results showed that photo-patterning of enzymes onto these substrates was a suitable technique for the implementation of multiple enzymes as long as they were patterned in the correct sequence. A summary of cascade reactions used for monitoring can be found in Table 2.

Table 2: Published enzymatic cascade reactions used for monitoring in continuous flow

<table>
<thead>
<tr>
<th>System</th>
<th>Enzymes</th>
<th>Product(s)/Reaction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme-Enzyme</td>
<td>Acetylcholine esterase, Choline oxidase</td>
<td>Bioelectrocatalytic reaction to determine AchE-inhibiting organophosphorus</td>
<td>(Han et al., 2012)</td>
</tr>
<tr>
<td>(Monitoring)</td>
<td></td>
<td>(1) β-Galactosidase, (2) Glucose oxidase, (3) Horseradish peroxidase (HRP)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Synthesis of fluorescent compound resorufin which is detected with inverted fluorescence microscope</td>
<td>(Fornera et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>(1) β-fructosidase, (2) hexokinase, (3) glucose-6-phosphate dehydrogenase, (4) phospho-glucose isomerase</td>
<td>Differentiation and quantification of sucrose, D-glucose and D-fructose</td>
<td>(Atalay et al., 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1) Candida antarctica Lipase B, (2) Glucose oxidase, (3) Horseradish peroxidase</td>
<td>Hybridisation of 2,2’-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1) Invertase, (2) Glucose oxidase, (3) Soybean peroxidase</td>
<td>Poly(p-cresol)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1) Invertase, (2) Glucose oxidase, (3) Horseradish peroxidase</td>
<td>Oxidation of Amplex Red by HRP from invertase-hydrolysed sucrose</td>
</tr>
</tbody>
</table>

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1.4. Microreactor Fabrication

Microreactor manufacturing has evolved greatly during the past few decades, with production methods and equipment becoming cheaper and more available for private or academic use, which allows a fast progression from design of a prototype to a finished reactor (Reichen et al., 2014). The most common methods for the production of a microfluidic channel or chamber are photolithography, etching, micro milling, laser ablation, powder blasting and hot embossing. These methods create channels in substrates with flat surfaces. Alternatively, additive deposition techniques, such as 3D printing can be used to. The choice of fabrication method is substrate and application dependent.

The most important factor when choosing a fabrication method is the consideration of the reactor material. Quartz, glass, silicone, polycarbonate and polymethylmethacrylate (PMMA) are among the most common reactor materials in use. In this section, the two reactor production methods used in this thesis are described, namely micromilling and laser ablation.

1.4.1. Photolithography

Photolithography is a commonly used variation of optical lithography and often used for the production of microfluidic devices. It is used to selectively shield a pattern on a silicone substrate by coating it with a photosensitive layer, referred to as a photoresist. The pattern is then developed with UV light. Two basic photoresist approaches can be used: the negative photoresist and the positive photoresist (Figure 3). In the positive photoresist approach, the exposed area is softened and removed. In the negative photoresist approach the exposed area is hardened and the shielded pattern is removed in the following step. Either etching or deposition can be used to create the desired pattern on the substrate during this step. Depending on the substrate material the photoresist can be bonded directly to create a microfluidic channel, or it can be used as a mould to create a negative image of the substrate. Moulding is commonly used for creating microchannel negatives for PDMS reactors. A disadvantage that comes with the
photolithographic process is that the substrate surface must be very clean. This method is usually performed in clean rooms or safety cabinets.

![Photolithography: Positive (left) and negative photoresist principle (right) (Geschke et al., 2004)](image)

**Figure 3: Photolithography: Positive (left) and negative photoresist principle (right) (Geschke et al., 2004)**

1.4.2. Etching

During the etching process, the non-shielded substrate is removed using chemical reactions. Hydrogen fluoride (HF) is commonly used for etching silicone dioxide. The reaction with pure HF occurs as follows:

\[
\text{SiO}_2 + 4 \text{HF} \rightarrow \text{SiF}_4 + 2 \text{H}_2\text{O}
\]

Due to the highly corrosive and toxic nature of HF, this fabrication method requires safety equipment and training. Etching can be used to produce structures down to the sub-micrometre scale, depending on the substrate and creates smooth channels. It can be used to produce glass microreactors and is commonly used in industry for the fabrication of bespoke reactors. Alternative methods that do not require the handling of strong acids are dry etching (either plasma etching or reactive ion etching) or the use of etching gases (Geschke et al., 2004).
1.4.3. Micro-Milling

The fabrication of microstructures through micro-milling occurs through the use of fast-spinning cutting tools of varying diameters to carve out the channels, inlets and other details of the reactor (Figure 4). This method is suitable for polymers such as polycarbonate and PMMA, as well as soft metals such as aluminium and can produce very smooth channels that allow for the optical analysis of the processes in the reactor using a microscope, optical fibre, or camera. With this method, channel widths down to micrometre dimensions can be created, depending on the milling tools used for the fabrication.

![Micro-machining tool drilling microstructures on aluminium block (ctemag.com, 2011)](image)

Figure 4: Micro-machining tool drilling microstructures on aluminium block (ctemag.com, 2011)

1.4.4. Laser ablation

Lasers can be used to focus a large quantity of energy on specific points in materials, creating very high localised temperatures. This causes materials to melt (glass) or evaporate (PMMA) in a desired pattern. The lasers are guided and focussed by an optical mirror system to create the pre-programmed patterns on a defined surface area. This method is very fast and allows for rapid prototyping. However, the channels that are produced via laser ablation are often not as smooth as the ones that can be achieved through micro-milling, depending on the laser system (Figure 5). This is due to the non-evaporated material pooling at the bottom of the channel once the laser has moved on and the material has cooled down. The laser can also leave raised ridges along the edges of the channel, which make the process of bonding less controlled and can lead to leakage in the reactor. A rather extreme channel profile of a laser fabricated channel with a
too-high intensity or too low speed can be seen in the figure below. A much smoother channel with a flat bottom can be achieved by correctly adjusting the intensity and speed of the laser. In the image below, the laser-ablated channel on the left shows an imprecise structure compared to the micro-milled channel on the right, which is square in comparison. The micro-milled channel also doesn’t show the molten ridges at the top of the channel where the material melted and cooled down in a small bump that will make the bonding to a lid less efficient.

Figure 5: Profile of a channel created in PMMA via laser ablation with a CO₂ laser as published by (Snakenborg et al., 2004) (left) and a micro-milled channel produced with a ball-pointed tool in aluminium (right) (Vázquez et al., 2013)

1.4.5. Powder Blasting

This method is used for the three-dimensional structuring of glass and brittle substrates. The substrate is covered with a mask to shield the areas that should remain unstructured. The masked substrate is then blasted with a high velocity powder beam. This leads to the erosion of the unmasked area and allows precise structures down to 100 µm. The structure of the created channels is uneven due to the nature of the method (Belloy et al., 2000).

1.4.6. Hot Embossing

For this method, a structure ‘embossing master’ is created, often via photolithography. The master is then used to stamp a channel pattern into a polymer that has been heated to its glass
transition temperature, thereby creating precise structures down to micrometre scale (Becker and Heim, 2000).

1.4.7. Fabrication Methods Used in this Work

In this thesis, laser ablation and micromilling were used to for the production of the microreactors due to their suitability and availability. In-house fabrication equipment made rapid reactor optimisation possible. Fabrication variables are detailed in the Materials and Methods section of each chapter. Once the reactors and channels have been created, considerations towards possible implementation of sensor technology can be made. From here on out the focus will be on optical sensor technology, due to the suitability of these sensors for monitoring enzymatic reactions. EUROMBR project partners at Graz University of Technology (TUG) possess advanced capabilities for optical sensor integration, suitable for pH monitoring, which were crucial for the enzymatic reactions studied in this thesis. Therefore, most of the sensor development presented in this thesis (Chapter 4) was undertaken during a secondment to TUG.

1.5. Introduction to Sensor Technology in Microfluidic Systems

The monitoring of key parameters such as oxygen, pH, carbon dioxide, glucose and temperature is essential for biotechnological processes. On a microfluidic scale, online monitoring is often a challenge since the miniaturisation of standard equipment is often difficult or not possible. Optical sensors are a good option for monitoring key analytes on both micro- and bench scale. According to the International Union of Pure and Applied Chemistry’s definition “A chemical sensor is a device that transforms chemical information, ranging from the concentration of a specific sample component to total composition analysis, into an analytically useful signal.” (Hulanicki et al., 1991). This definition applies to all sensor types regardless of their measurement principles, though the focus in this work is on optical sensor formats only.
Lübbers et al. (Lübbers and Opitz, 1983) and Wolfbeis et al. (Wolfbeis et al., 1985) first demonstrated the oxygen sensing principle based on fluorescence quenching of immobilised dyes in the presence of oxygen. They used various fluorescent dyes embedded in polymers to produce the first optical oxygen sensors. This sensing principle has since grown in popularity due to the sensor’s low cost, compact size and applicability in closed systems. The principle is non-invasive and does not consume the analyte. These sensors are now used for a wide variety of applications including environmental monitoring (Clarke et al., 2017), bioprocess monitoring (Demuth et al., 2016) and in life sciences (Wang and Wolfbeis, 2014, Demuth et al., 2016). Real-time oxygen monitoring was also at the core of establishing microfluidic bioreactors as a field of research, due to their need for monitoring growth in microliter-to-litre volumes (Kirk and Szita, 2013, Schäpper et al., 2009).

Due to the successful implementation of optical oxygen sensors in various systems and applications, sensors for more analytes were developed. Luminescence principles were used to develop sensors for parameters relevant for bioprocess monitoring, such as pH, temperature, glucose and carbon dioxide. Optical sensors are very suitable for microfluidic applications in biological systems. Their potential for miniaturisation, in situ real time monitoring capabilities and non-invasive principle can be an alternative to the more traditional electro-chemical monitoring principles, such as electrodes, which are often not suitable for miniaturisation to microfluidic channel dimensions. For example, oxygen sensors in contrast to the Clark electrode, their electrochemical counterpart, do not consume the analyte.

The Severinghaus electrode (Severinghaus and Bradley, 1958), used for carbon dioxide sensing, is difficult to adapt and integrate to microfluidic feature sizes because it requires a buffer reservoir for reference. Furthermore, the detection unit is not necessarily mechanically attached to the device (as opposed to electrodes). This enhances the flexibility of operation for microfluidic devices, which makes the optical sensor a preferable choice for microreactor systems (Sun et al., 2015, Pfeiffer and Nagl, 2015).
Sensors usually consist of a component that interacts with the analyte, such as a dye or antibody, a transducer that translates the interaction of the sensing component with the analyte into an electrical signal and a signal processing unit that translates that input into an output that can be processed by a computer. An ideal sensor should be cheap to produce, fast in response, selective to a single analyte and user-friendly (Göpel, 1992). Other parameters, such as stability and the limit of detection, need to fit the application. In processes ranging from microfluidic scale to industrial scale different modes of measurement are available depending on the monitoring requirements, the main modes being online and offline monitoring. The difference is that online measurement is achieved via a sensor in the production flow, while offline measurement occurs via sample taking and analysing the sample outside of the production flow. The latter method makes rapid process control impossible as there is no real-time feedback about the system. However, they pose less risk for contamination compared to measuring online as they are non-invasive.

To summarise the advantages and disadvantages for each measurement mode and further sub-classifications of the terms, the following table has been created to highlight the advantages and disadvantages of different methods of monitoring based on the principles of process analysis published by Kessler (2006), which is aimed at larger-than-micro scale processes.

Monitoring with optical sensors in microfluidic application usually occurs online (which is often synonymous with in-line and in situ for microfluidic devices) or at-line. The term ‘online’ implies that the sensors are integrated inside the flow path, are in direct contact with the reaction and that the sensor signal is read out in real-time and in an automated manner (in-line monitoring on the other hand can be manual or a discontinuous, non-automated readout). At-line monitoring means that the measurements are taken outside of or at the end of the flow path. While online monitoring allows the generation of a time-course reaction profile under the variation of flow rate (or the implementation of measurement at multiple reactor positions as shown in Chapter 5) inside the reactor, at-line monitoring provides endpoint data. Further
benefits of online monitoring as well as downsides in comparison to at-line and offline monitoring are summarised in Table 3.

Table 3: Summary of advantages and disadvantages of the different monitoring modes (from Gruber et al. 2017 (Gruber et al., 2017c))

<table>
<thead>
<tr>
<th>Summarised</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| Online     | - Real time analysis possible  
- Rapid feedback allows real time process control  
- No manual sampling required  
- Measurement at real temperature  
- Less risk of contamination  
- Production flow undisturbed by sampling or redirecting | - Possible interaction of sensors with the flow or reactants  
- Sensors need to be recalibrated and replaced over time  
- Increase of system complexity (fabrication, design, operation, maintenance)  
- Cross sensitivity with other analytes or interferences can be difficult to quantify  
- Limited to a specific analytical problem and a certain concentration range |
| At-line    | - Significant number of assays/analytical methods available  
- Can be cost-effective  
- Flow cells available  
- Feedback available quickly | - Changes in sample before analysis possible  
- Analysis limited to on-site equipment  
- Certain sample volume needed  
- Risk of contamination through sampling |
| Off-line   | - Versatile component analysis possible through instrumental analytical tools (LC/GC/MS)  
- Variety of analytes can be monitored with one analytical method  
- Availability of analytical methods with high selectivity and accuracy | - Slow feedback  
- Results not in real-time  
- Risk of contamination through sampling  
- Sample degradation possible  
- Analysis at the end of process |

In the following sections, an overview of optical sensor formats, fabrication and integration will be given, and the considerations necessary for a successful implementation into a system will be delineated. Following that, the principles of the different detection methods will be discussed. Overall, it is important to consider the development of monitoring methods for any given system as an iterative process.

1.5.1. Optical Sensor Formats

The final state of a sensor is described by the term format, meaning the physical state of the final integrated sensing material. The aim of sensor integration is a robust readout and sensor viability for the duration of the application. Whether a sensor format is suitable for a given
application is dependent on whether sufficient signal intensity for readout can be achieved after
the whole system has been assembled. Further considerations are the suitability of the dye for
the reactor material; for this, material transparency and material-dependent cut-off wavelengths
must be considered. For biological applications, considerations of cell toxicity and feasibility
of sterilisation between uses must also be considered.
In the following sections, the most common sensor formats used in microfluidic devices are
discussed in more detail, and considerations for their compatibility with different reactor
materials and bonding methods are discussed.

1.5.1.1. Dissolved Indicators
In terms of sensor integration, the use of dissolved indicator dyes is the most easily
implemented principle. The dye is dissolved in the mobile phase of the reaction or pumped into
the reactor alongside the reagents (Figure 6 A). The dye is not embedded in a matrix, so the
response time is very fast. However, this means that the indicator dye molecules are present in
the product stream and require an additional downstream processing step to be separated from
the product. The possibility of an interaction between indicator and reactants is prevalent,
meaning the reagents could destroy the dye, or enzyme could be inactivated due to dye
presence, or cell toxicity could occur. The application of these dyes in flow can be difficult
when compared to their use in a stirred batch system. This is due to the shorter optical path
lengths in microreactor channels, often yielding insufficient signal for detection. Increasing the
concentration of the indicator to achieve the desired signal is often not possible because many
dyes form aggregates, which leads to inhomogeneity and makes the readout unreliable. This is
only an option for simple systems that do not require sophisticated measurement principles.
Optical pH indicator dyes for example have been used to determine the pH gradient in systems
(Lee et al., 2008). A completely homogeneous distribution of the indicator dye and an
unchanging background must be established in order to achieve a reliable readout. It should
also be noted that this approach is only suitable if the reaction mixture is not turbid, or the turbidity of the reaction mixture is constant and can be compensated for with calibration.

Figure 6: Different sensor formats in a microfluidic channel (grey): a) Dissolved indicator dye in the entire channel (red) b) Sensor layer in the form of a homogeneous film (red) c) sensor layer in the form of sensor spots d) Free sensor particles and e) magnetic sensor particles. The liquid phase is represented in blue. Adapted from Sun et al. (2015) (Sun et al., 2015) and published in Gruber et al. 2017 (Gruber et al., 2017c)

1.5.1.2. Sensor Layers and Sensor Spots

A stationary sensor that remains in the reactor is usually preferable for biotechnological process monitoring, since it does not require a downstream removal of indicator dyes. To immobilise sensing dyes, they are usually embedded in a polymer matrix, either through covalent bonding or entrapment into the matrix. The polymer needs to be chosen according to the application and sensing dye. Ideally, it should be permeable to only the analyte and not the other components or possible contaminants in the sample. It is crucial that the polymer is inert to the analyte as well as the rest of the reaction mixture. The choice of lipophilic versus hydrophilic host polymers is dependent on the analyte and facilitates the interaction between the analyte and the
sensing dye. Oxygen sensors require a lipophilic host polymer while pH sensors are usually entrapped in a hydrophilic matrix.

Stationary sensors are usually integrated in the form of sensor layers or spots (Figure 6 B, C). Using this sensor format, the analyte can be detected at multiple and different positions in microfluidic channels or reaction chambers. Sensor layers enable the imaging of a large area in order to visualise gradients in a channel. The most common integration methods such as photopolymerisation, screen-printing, spray-coating, spin-coating and gluing of sensor foils will be discussed later in this chapter.

Sensing layers, both in spot and foil format, are commercially available for oxygen, pH and carbon dioxide; Dissolved oxygen sensors in the range of 0-100% air saturation and trace level detection are available from Pyro-Science and PreSens (Pyro-Science, 2016, PreSens, 2015). Also available are pH sensors in the range of pH 5.5-8 (PreSens, 2015, Ocean-Optics, 2016) and sensors carbon dioxide in the range of 1-25% CO₂ at atmospheric pressure (PreSens, 2015). They are usually rather large ranging from 3 to 8 mm. This size is often not viable for integration into microreactors with small channels. Creating sensor chambers to accommodate chambers of this size can lead to dead zones, undesired flow patterns and create air bubble traps. For channels in reactors like the ones used in this thesis, smaller sensor spots needed to be developed, but for microbioreactors with large chamber, these sensor sizes may be suitable as shown by Zanzotto et al. (Zanzotto et al., 2004), Super et al. (Super et al., 2016) and Kirk and Szita (Kirk and Szita, 2013).

1.5.1.3. Sensor Beads or Particles

One way to avoid strain on the sensor matrix and dye through thermal interactions during the bonding is to add the sensors after the microreactor is fully assembled. This is possible through the use of sensor nano- and microbeads and sensor particles. As a result, the sensor particles are flushed into the microreactor after it has been fully assembled. This allows the use of
thermally unstable dyes. The use of particles can enable the integration of more sensor dye and allow a brighter signal for a better readout because the polymeric material can accommodate a higher dye concentration without exhibiting self-quenching effects (Figure 6, D). Nanoparticles or micro- and nanobeads can be introduced into the reactor after assembly and sterilisation. The use of pillars and other secondary support structures in the chip makes it possible to accumulate enough sensor material in one spot to achieve a sufficient readout. It also makes recovering the sensor particles possible by back-flushing them out of the system. Different mechanisms have been used for this purpose, be it optical tweezers (Klauke et al., 2006) or pillar-like structures within the channel that catch the beads and keep them from being carried away by the fluid flow. This way, particles can be accumulated in one spot to create sufficient signal intensity. Micro-particles are commercially available for oxygen monitoring (Pyro-Science, 2016, Colibri-Photonics, 2016). The use of magnetic optical sensor particles, or MOSePs, as introduced by Mistlberger et al., (Mistlberger et al., 2009), which are held in place with a small magnet, allows the formation of sensor spots in situ at the desired readout location.

The advantages and disadvantages of the presented sensor formats have been summarised in Table 4.
Table 4: Advantages and disadvantages of the most common sensor formats

<table>
<thead>
<tr>
<th>Sensor format</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissolved indicator dye</td>
<td>- Easy to implement for simple measurements</td>
<td>- Indicators have to be added and removed from sample</td>
</tr>
<tr>
<td></td>
<td>- Spatial analyte imaging possible (but requires a suitable detection system)</td>
<td>- Homogeneous dispersion of dye required for accurate readout</td>
</tr>
<tr>
<td></td>
<td>- Universally applicable, largely independent of reactor design</td>
<td>- High concentrations necessary for signal strength</td>
</tr>
<tr>
<td></td>
<td>- Application after device bonding</td>
<td>- Potential interference with sample</td>
</tr>
<tr>
<td></td>
<td>- Readout anywhere in device</td>
<td></td>
</tr>
<tr>
<td>Layers or spots</td>
<td>- Stationary</td>
<td>- Can be difficult to integrate</td>
</tr>
<tr>
<td></td>
<td>- Easy to use once integrated</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- No separation steps necessary</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Interference with sample unlikely</td>
<td></td>
</tr>
<tr>
<td>Beads or particles</td>
<td>- Ease of use once developed</td>
<td>- Particles have to be added and removed from sample</td>
</tr>
<tr>
<td></td>
<td>- Application after device bonding</td>
<td>- Homogeneous dispersion of particles required for accurate readout</td>
</tr>
<tr>
<td></td>
<td>- Flexibility in reactor design and production</td>
<td>- Poor stability of the suspension of particles can lead to inhomogeneity</td>
</tr>
<tr>
<td></td>
<td>- Readout anywhere in device</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Less interference with sample compared to dissolved indicators</td>
<td></td>
</tr>
</tbody>
</table>

1.5.2. Deposition Techniques for Sensor Layers

An overall challenge is finding sensors that are both capable of emitting enough signal to reliably detect changes in analyte, as well as ones that can cope with the low analyte amounts available in microscale systems. Of course, the sensitivity and specificity towards any given analyte must be sufficient as well. The method of integration is a crucial part in achieving signal strength suitable for the system. Depending on the microreactor material, surface treatment is necessary in order to support and retain the sensor material. How fast a sensor achieves a steady signal depends on multiple parameters, such as the reactor dimensions, sensor thickness and flow rate.

1.5.2.1. Direct Staining of Device Material

Permeable materials such as PDMS can be stained with sensing dyes, thereby transforming the reactor material itself, whole or in part, into a sensor. Challenges to consider for this method are the readout and the polymer permeability. This method has found use in oxygen
(Skolimowski et al., 2010) and temperature (Zhou et al., 2009, Samy et al., 2008, Gui and Ren, 2008) sensing in microfluidic systems previously. It is also possible to stain membranes with this technique and then insert the membranes into the reactor.

1.5.2.2. Spin- and Knife-Coating

The aim of the spin coating technique is to achieve a sensing film of homogenous thickness. It can be used to produce optical sensing films down to a size of a hundred nanometres (Ungerböck et al., 2013a, Chrimes et al., 2013). For this purpose, the sensor dye is suspended in a matrix usually consisting of the host polymer and a solvent. This ‘cocktail’ is applied to an inert smooth surface and spun in order to spread the fluid evenly using rotational force. The film is left to dry and later removed and is then integrated into the microreactor. A potential downside of this technique is material waste during the production process.

Similarly, during the process of knife coating the sensor dye in matrix dissolved in solvent is applied onto its intended surface and spread into an even layer using a ‘knife’ which produces a defined gap between the surface and the knife and thereby results in an even film. Polymer foils and glass slides are common carriers for knife-coated sensors. This method is very easy, but just like spin coating it requires structuring of the obtained sensor film afterwards. For this purpose, spots are usually cut from the obtained foil, which then have to be glued into the reactor (Ungerböck et al., 2013a). Grist et al used this technique to create a sensor film which they then modified using laser ablation (Grist et al., 2014).

1.5.2.3. Screen-Printing

For this method, a mesh that is coated with a sensor dye-impenetrable blocking stencil is applied to the area of the reactor that should remain free from sensing materials and the remaining area is coated in sensing matrix after which the stencil is removed, leaving the areas that were covered free of material. This method can be used to produce spots or layers on flat surfaces in
a size as small as 100 µm depending on mesh and ink composition and feature sizes (Metters et al., 2011). The downside is that much material is wasted in the process, which can be a limitation depending on the value of the sensor matrix used. Another disadvantage is that a completely flat channel surface is required for the process as the application into grooves is not possible. It is most suitable to apply the mesh to the lid of the channel and bond the structured layer of the reactor to the sensor-coated lid.

**1.5.2.4. Photo-Polymerisation**

Photo-polymerisation is used for the production of small sensors with high dimensional accuracy. Photomasks are used to expose only the desired area of a photoresist matrix containing sensing dye to UV light. The matrix, usually poly(ethylene) glycol (PEG), hardens under the UV exposure and creates the sensor spot (Zhan et al., 2002) (Koh and Pishko, 2005). Moulds of the desired sensor dimensions can also be fabricated in another polymer, such as PDMS. It is then filled with a luminescence dye-doped photoresist which is then cured. Finally, the PDMS is removed to only leave the sensor shape behind.

**1.5.2.5. Spray-Coating**

This technique requires the sensor dye to be dissolved in a solvent and host polymer matrix, after which it is sprayed onto the desired substrate, such as spraying the channel or reactor bottom. This method can be used in combination with stencils to only cover the necessary areas. The sprayed layers can be sized down to 100 µm with stencils (Ehgartner et al., 2016b, Rennert et al., 2015).

**1.5.2.6. Microdispensing and Inkjet Printing**

Microdispensing or inkjet devices are used to deposit ink droplets via thermal, piezoelectric, acoustic, electrostatic, electro-hydrodynamic actuation, or valves (Li et al., 2015). A guided
tappet is used to propel a droplet of the sensing matrix from a reservoir through a nozzle. This method is particularly suitable for viscous liquids and sensor matrices that contain sensing nanoparticles and has been used for the integration of pH sensors (Herzog et al., 2014, Tahirbegi et al., 2017, Gruber et al., 2017d). The dimensions of the sensor layer are determined by the viscosity, nozzle size and number of layers sprayed onto the same area. The luminescence intensity is directly dependent on these parameters, as well as the sensing dye concentration.

1.5.3. Sensor Integration and Microfluidic Device Sealing

Once the reactor’s structure has been produced and a sensor has been integrated using any of the methods above, the microfluidic channels and chambers must be sealed with a lid to close the reaction system. Multiple processes can be used to achieve this; the ones used for the duration of this thesis are briefly described here. It is important to consider possible bonding methods ahead of time and coordinate them with the sensor deposition technique and sensing material. Some sensor material and dyes cannot withstand exposure to high temperatures, while some dyes might bleach under the exposure to UV light. Chemical bonding methods can lead to a reaction with reactor material. Therefore, the following methods must be considered for their potential impact on integrated sensors.

1.5.3.1. Thermal bonding

Thermal bonding is a process suitable for polymer reactors. It is a direct bonding method, meaning that it connects to substrates of the same composition (Nguyen, 2002). It is used to irreversibly bind the two substrates (structured reactor and lid) together. For this purpose, the two are pressed together mechanically at a constant pressure and heated to the glass transition temperature of the substrates which leads to a physical linking of the two layers that persists when they cool down. To avoid leakage within the reactor, the layers must be fully connected.
to each other in the area surrounding the microstructure. In terms of sensor integration, it is important to consider the sensing material’s tolerance of high temperatures. Commercially available oxygen sensors tend to be thermostable up to 120 °C and as a result also suitable for autoclavage, while for other analytes, this is dependent on the sensor composition on a case by case basis. The pH sensors used in this work proved to be thermostable and could be used in PMMA reactors bonded at 110 °C for 45 min (Gruber et al., 2017d) while retaining excellent sensitivity.

1.5.3.2. Plasma-Assisted Bonding

Plasma-assisted bonding uses cold plasma to activate the surface of the reactor material in order to create an irreversible bond when the second substrate, usually the lid, is applied. This allows the bonding of two substrates of different compositions at low temperatures (Xia and Whitesides, 1998). This method is often used for reactors that include moulded PDMS components.

This is a low-temperature process, but when sensors are integrated into the reactor the plasma can still affect the sensor composition. This does not mean that the method is not suitable for reactors containing sensors, Nock et al. (Nock et al., 2008) used plasma-assisted bonding to bond their sensor dye-stained PDMS lid to the rest of the reactor material.

1.5.3.3. Clamping

For this method, the reactor and the lid are only connected via the mechanical pressure applied by clamps (Reichen et al., 2014, Tkachenko et al., 2009, Brevig et al., 2003, Tamanaha et al., 2009). This means that assembling a leak-free system can be difficult and usually requires a custom-made clamp system. The reason why it is still a common method is because the microreactor structure is accessible at any time, which is especially relevant to cell culture applications (Reichen et al., 2014). In terms of sensor integration, clamping systems are
especially useful because they are compatible with all sensing types and require no thermal or chemical bonding that could damage a sensor. Clamping systems are also commercially available (Viehues et al., 2017, Micronit, 2015).

1.5.3.4. Adhesive Bonding
Adhesive bonding is the process of using an intermediate layer to connect the two substrates to each other. This can be a photoresist, glass, tape or different polymers (Nguyen, 2002). Often, UV-curable glue is used to connect the reactor channel and lid to each other. The glue is liquid and easy to handle until it is exposed to UV light, hardening the glue and thereby binding the two substrates (Chiang et al., 2016). It is important to consider that sensing dyes are prone to bleaching under prolonged exposure to UV light.

1.5.3.5. Solvent-assisted Bonding
For thermoplastic polymers, an alternative bonding measure is solvent-assisted bonding. In this process, the polymer surface is softened using an organic solvent prior to compression bonding, or thermo-compression bonding. In some cases, the softening alone can be sufficient to facilitate a permanent bond, in others it can be used to lower the bonding temperature. This could be a useful trick for bonding reactor parts that contain thermosensitive sensor material. However, the presence of organic solvent fumes can be as damaging, or more so, than thermal bonding. In smaller structures, solvent splashes and solvent fumes could deform the reactor material and clog the reaction channels (Ng et al., 2016).

1.5.4. Luminescence Detection Principles
The term ‘photoluminescence’ encompasses the phenomena of fluorescence, phosphorescence and delayed fluorescence. Optical sensors are, for the most part, based on photoluminescence. They are usually high in signal intensity and are analyte specific due to the defined emission
spectra of luminescent dyes, which means that interference with reagents and other sample components is unlikely (Valeur, 2001). The sensor format, measuring principle and readout methods are application-dependent. Integrated readout techniques, consisting of a detector, a light source and a sensor in contact with the reaction solution are among the most popular readout methods, alongside fluorescence microscopy and optical fibres. Spectrophotometric determination of the sample absorption is also a common method, though rarely used for microfluidic applications. These common detection principles will be briefly introduced in this section. Figure 7 demonstrates the principles visually:

Figure 7: Schematic representation of detection principles (a) Luminescence lifetime determination by time-correlated single photon counting (TCSPC). (b) Lifetime determination by gated detection: rapid lifetime determination shown. (c) Lifetime determination by phase modulation. (d) Dual wavelength ratioing. Reproduced from (Bolivar et al., 2013)
1.5.4.1. Intensity-Based Detection

The luminescence detection based on intensity is the most easily realised measurement technique; the changes can be detected using a fluorescence microscope. There are a few considerations to be made to ensure a reliable readout, namely, the luminescence intensity is sensitive to variations in the light source and interference with ambient light, as well as fluctuations in the detector. In this application the homogeneous distribution of the sensing dye is of paramount importance, as aggregation in the matrix can lead to false results. Photo-bleaching of the dye and light scattering are also a concern. Due to this reason, lifetime measurement and ratiometric detection are usually preferable.

1.5.4.2. Single-Photon Counting

(Time-correlated) single-photon counting is used to measure the excited state lifetime. For this purpose, single photons are counted after excitation with a laser pulse using a photodetector. The number of detected photons and the time measured between excitations is recorded in a decay curve, from which the lifetime can be determined using an exponential fit function (Figure 7 a). Decay times down to a nanosecond range can be determined using this method. This method is not usually used for online monitoring in microfluidic system, due to the complexity of the required setup. So far, this principle has only been demonstrated for temperature monitoring in a microscopic imaging setup by Bennet et al. (Bennet et al., 2011).

1.5.4.3. Time-Gated Detection

This method also measures the lifetime of a luminophore, which is excited by a laser pulse. After that, the emission is recorded in two or more ‘time gates’, allowing the monitoring to be conducted in intervals (Figure 7 b). The ratio between the image intensity detected in each of the timed gates is used to determine the lifetime (Sytsma et al., 1998). This method has been
used to determine the lifetime of oxygen-sensitive dye in a PDMS reactor for cell culture (Sud et al., 2006, Molter et al., 2009).

1.5.4.4. Phase Modulation

Using phase modulation, the luminescent lifetime is determined in the frequency domain, rather than the time domain. The dye is excited with amplitude-modulated light, for example in a sinusoidal function (Figure 7 c). The resulting modulated emission has a decay time that depends on the lifetime of the luminophore. The delay between modulated responses can be measured as a phase shift and used to calculate the lifetime of the luminophore (Zanzotto et al., 2004, Ehgartner et al., 2016b). This method is suitable for luminophores with lifetimes larger than one microsecond and requires only inexpensive instrumentation the size of a memory stick as used by Ehgartner et al. (Ehgartner et al., 2016b). This method has been used to monitor oxygen in microfluidic devices (Zanzotto et al., 2004, Ehgartner et al., 2016b). Dual lifetime referencing is a subcategory of this method in which an analyte-sensitive dye and a reference dye are measured for their decay time using fibre optic readout. This method is frequently used for pH sensing and is the readout used to measure the pH in this thesis (Zanzotto et al., 2004, Tahirbegi et al., 2017, Gruber et al., 2017d).

1.5.4.5. Two Wavelength Ratioing

In this method, two bands in the emission spectra are monitored at the same emission wavelength, or, alternatively, a reference dye is added if the sensing dye does not show two emission bands that can be separated reliably. Then the ratio of the two signals is determined for various analyte concentrations through calibration. This makes miniaturisation difficult, since sufficient signal intensity is difficult to achieve, however, colour CCD cameras have been demonstrated for the application with microfluidic devices in the past (Ungerböck et al., 2013a).
1.5.5. **Summary**

A number of potential hurdles have been pointed out for both chemo-enzymatic and enzyme-enzyme cascades, such as product degradation, reduced enzyme activity through substrate or product inhibition, deactivation of enzymes by solvents and non-compatible reaction conditions. A framework for optimising coupled cascade reaction, in terms of guidelines and consideration on how to overcome the key issues, needs to be devised. The implementation of real-time monitoring through optical sensor technology can give valuable insight into the reaction progress in a microfluidic reactor. The feedback from sensors can be used to intervene if poor reaction conditions are detected. Insights into necessary considerations for sensor formats, as well as sensor integration and detection have been given. These considerations will be used in the following chapters to meet the aims and objectives of the project.
1.6. Aims, Objectives and Thesis Structure

1.6.1. Aims and Objectives

This project aims to produce guidelines for developing cascading reactions at a microfluidic scale by using two cascade case studies, one chemoenzymatic reaction system and one enzyme-enzyme reaction system. Microfluidic reactors and reactor cascades capable of accommodating the overlap of the windows of operation for each reaction step will be designed. Online monitoring will be used to gain insight into the reaction progress inside the microreactors. The hypothesis that will be tested is that online monitoring will enable better process control of cascading enzymatic or chemo-enzymatic reactions and thus improve conversion rates and yields at the microfluidic scale. In order to achieve this, the following objectives will be addressed:

• Development of microreactors suitable for chemoenzymatic and enzyme-enzyme reaction cascades, capable of accommodating different flow rates and reaction conditions as necessary for each cascade.

• Development of optical sensors suitable for integration into microfluidic reactors for process monitoring. This requires sensors with sufficient sensitivity, stability and brightness, as well as a method of integration for the developed sensors into the reactors and a reactor bonding method that does not damage the sensors.

• Reaction progress monitoring in the developed reactor, using the integrated sensors and demonstration of improved process control.

• Development of strategies for optimisation of the cascading reactions.

1.6.2. Model Systems and Rationale

A microfluidic approach will be applied to continuously produce pharmaceutical precursors under real-time monitoring of process variables. The monitoring will be conducted using optical sensors since regular electrodes and detectors are not suitable for the microfluidic scale.
Optical sensor technology offers the possibility of real-time quality assurance and process monitoring, which is crucial for reliable reaction control. The model reaction systems studied for this purpose are: a chemo-enzymatic cascade (Diels-Alder reaction followed by a transketolase reaction) and a two-enzyme (transketolase reaction followed by a transaminase reaction) cascade system. Free enzymes are used in both reaction cascades. Both reaction schemes include a reaction catalysed by transketolase with hydroxypyruvate as a co-substrate. Due to the choice of co-substrate of the reaction, carbon dioxide is formed as a side-product. This leads to a shift in the reaction equilibrium towards the products side and accounts for the irreversibility of the reaction. Additionally, no other side-products are formed, which makes subsequent separation steps redundant and potential product purification steps easier. However, the increase of carbon dioxide can lead to a pH increase in the reaction mixture, which is why online monitoring is crucial for a successful conversion and continuous synthesis of the intended compounds. Miniaturised optical pH sensors will be integrated into the microfluidic devices to monitor the reaction progress.

1.6.3. Thesis Structure

The thesis is structured as follows: Chapter 2 and 3 will detail the work on the two model cascades. Chapter 4 will focus on the development of the sensor technology needed to monitor the reactions and show the development of suitable pH sensors. It also contains preliminary work on the development of novel carbon dioxide sensors. Chapter 5 shows the application of a microfluidic side-entry reactor into which the pH sensors were integrated. Each chapter is divided into an Introduction section followed by Materials and Methods, a Results and Discussion section and ends with a Conclusion and Suggestions for Future Work.
2. Optimisation of 2-aminobutane-1,3,4-triol Synthesis through Transketolase - Transaminase Coupling in a Microfluidic Cascade Reaction

2.1. Introduction

In this chapter a continuous flow two-enzyme system, anchored on microscale technology, aimed at the production of industrial relevant synthons, namely chiral amino-alcohols, was developed. Chiral amino-alcohols are key industrial synthons for the production of optically pure pharmaceuticals, such as broad spectrum antibiotics, like chloramphenicol and thiamphenicol (Bhaskar et al., 2004; Boruwa et al., 2005), HIV protease inhibitors (Kaldor et al., 1997; Kwon and Ko, 2002) and active molecules such as (S)-amphetamine (Rozwadowska, 1993). In fact, it is estimated that up to half of all pharmaceutical drugs contain a chiral amine in its structure (Ghislieri and Turner, 2014; Zhu and Hua, 2009). The chemical routes for the production of these compounds are usually complex and necessitate multiple steps, employing expensive transition metals catalysts and leading to low productivities (Ghislieri and Turner, 2014; Tamura et al., 2014). Most syntheses protocols require intermediate purification steps for which large amounts of organic solvents are used, especially when multiple chromatography steps are necessary. If a continuous multi-step cascade could be optimised to achieve a series of reactions while eliminating the need to purify intermediate products, a great reduction in process costs and overall process time could be achieved. At the same time the elimination of intermediate purification steps would lead to an overall improvement of the ecological footprint of the process.

Owing to their environmental benign nature and high selectivity, biocatalytic syntheses of chiral amino-alcohols have been shown to be a robust alternative to conventional chemical routes (Höhne and Bornscheuer, 2009; Wohlgemuth, 2010). Compared to several enzymes employed in the synthesis of chiral amino alcohols, such as imine reductases (Leipold et al., 2013), amino acid dehydrogenases (Zhu and Hua, 2009), transaminases, lyases and monoamine
oxidases (Ghislieri and Turner, 2014; Höhne and Bornscheuer, 2009)), transaminases present a broader substrate specificity and higher regio- and stereoselectivity. However, their application is hindered by unfavourable equilibria and substrate and/or product inhibition (Rios-Solis et al., 2015; Stewart, 2001; Taylor et al., 1998; Villegas-Torres ., 2015). This is especially the case when the enzyme is coupled with additional chemical or biocatalytic reaction steps.

In general, the majority of the reported biocatalytic processes and biotransformations in microreactors have focused on the use of a single enzyme catalysing a given reaction. However, when mimicking what occurs in living cells, more complex compounds can be synthesised via multiple enzyme reactions, in which the product of one reaction serves as substrate in the subsequent reaction. The use of multiple enzyme reaction schemes avoids the time-consuming and/or yield-reducing isolation and purification of intermediates, that can be very unstable, (Smith et al., 2010) and allows the use of less expensive substrates, leading to an overall reduction of costs, energy and auxiliary chemicals consumption which ultimately leads to a greener process (Bruggink et al., 2003; Findrik, 2009; Lopez-Gallego and Schmidt-Dannert, 2010; Schrittwieser et al., 2011). In order to establish the guidelines for the optimisation of cascading enzymatic reaction system, a well-known enzymatic cascade, was selected. The biocatalytic de novo chiral-amino alcohol synthesis presented in this chapter involves the coupling of a transketolase (TK)-catalysed asymmetric carbon-carbon bond formation with a transaminase (TAm)-catalysed reaction of the keto-group into a chiral amino group (Figure 8) (Ingram et al., 2007; Rios-Solis et al., 2011) for the production of (2S, 3R)-2-amino-1, 3, 4-butantetriol (ABT). During the first biocatalytic step the achiral substrates hydroxypyruvate (HPA) and glycolaldehyde (GA) are converted into L-erythulose (ERY) by wild-type Escherichia coli TK and subsequently Chromabacterium violaceum 2025 o-TAm catalyses the reaction between ERY and the amine donor (S)-a-methylbenzylamine (MBA) to yield ABT and acetophenone (AP) as a by-product. ABT has been used as a building block for statins employed in the synthesis of the HIV-protease inhibitor Nelfinavir (Kaldor et al., 1997; Kwon
and Ko, 2002) and as an intermediate in the synthesis of detoxinine, a detoxifying agent for reducing the toxicity of the antibiotic treatment for rice blast disease (Ingram et al., 2007; Monache and Zappia, 1999).

Figure 8: Reaction scheme for the transketolase-transaminase-catalysed synthesis of (2S, 3R)-2-amino-1, 3, 4-butanol. PLP: pyridoxal-5’-phosphate, ThDP: thiamine diphosphate

The production of chiral amino-alcohols employing a multi-enzyme system has been performed with whole cells and enzymes (purified or as cell extract) (Ingram et al., 2007; Rios-Solis et al., 2011) in the same reaction space (one-pot systems), where the biocatalysts and chemicals are added simultaneously (Ingram et al., 2007), or in sequential mode (fed-batch) (Rios-Solis et al., 2011; Villegas-Torres et al., 2015); or, alternatively, in separate reactors (Smith et al., 2010). The use of purified enzymes or lysates reduces the reaction complexity, allowing an easier control over the operational conditions and biocatalyst concentrations and overcomes the often-limiting transport of substrate and product through the cell membrane encountered when using whole cells. However, purified enzymes were revealed to be less stable under operational conditions when compared to enzyme lysates (Ingram et al., 2007). Likewise, in whole-cell systems the enzyme stability is in need of improvement (Ingram et al., 2007; Rios-Solis et al., 2011). Operation in one-pot sequential mode (fed-batch, or sequential batch) or in separate reactors has proven to be preferable over one-pot with simultaneous addition of reagents and biocatalyst, since it prevents substrate/product cross-inhibitions and decreases the occurrence of undesirable side-reactions (Ingram et al., 2007; Rios-Solis et al., 2011; Smith et al., 2010; Villegas-Torres et al., 2015). Nonetheless, when performed in separate reactors, the isolation
of the intermediate product led to a loss of more than 30% mol/mol (Smith et al., 2010), therefore a continuous reaction where the intermediate is immediately used in the second reaction step is preferable. The use of modular microfluidic reactor units could be the right approach to successfully accomplish this task.

Previous studies have shown the suitability of performing the TK-TAm coupled reaction in structured microfluidic reactors employing immobilised biocatalysts (Matosevic et al., 2011a)(Abdul Halim et al., 2013); nonetheless, it was observed that the immobilisation protocol and mass transfer constrain enzyme performance, resulting in prolonged reaction times. The biocatalyst performance can be hindered by the immobilisation, especially if non-specific attachment occurs, such as the active site being inaccessible due to the enzyme’s orientation against the substrate onto which it is immobilised.

In the present work, a TK-TAm coupled reaction process using free enzymes in microfluidic continuous reactors was developed, investigating the capability of microfluidic reactors to aid in vitro multi-enzyme biocatalytic process evaluation and intensification.

Aiming at reducing the time scale of the coupled reaction, increasing biocatalyst concentrations in the form of lysate were employed in both reactions. A maximal concentration of 3.25 TK U mL\(^{-1}\) was employed in the transketolase step. This allowed full substrate conversion in less than 15 min; whereas for the transaminase step a concentration of 10.80 TAm Units mL\(^{-1}\) resulted in a reaction time of 2 h and achieved 100% conversion. The obtained results are illustrative of the use of continuous flow microreactors as a useful tool for the design and evaluation of multistep biocatalytic conversions employing enzyme lysates. The key results presented in this chapter have been published under the title ‘Enzymatic synthesis of chiral amino-alcohols by coupling transketolase and transaminase-catalysed reactions in a cascading continuous-flow microreactor system’ in Biotechnology & Bioengineering (Gruber et al., 2017a).
2.2. Materials and Methods

Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich (Gillingham, UK) and were used without further purification. Unless otherwise stated, reactions were performed and analysed in triplicates.

2.2.1. Fabrication of Microreactors

All components of the microreactor (Serpentine bioreactor) were designed using Solidworks® (Dassault Systems, Velizy-Villacoublay Cedex, France). The channels and cut-outs were fabricated from 1.5 mm poly(methylmethacrylate) (PMMA) using a CO$_2$ laser marking head (Laserlines, Banbury, UK). The layers were thermo-compression bonded (50 min, 105 °C min). The interconnect blocks of the microreactor were fabricated in 5 mm PMMA with a micromilling machine (Folken, IND, USA), using a 2 mm diameter end mill with a spindle speed of 10,000 rpm. M3 and M6 taps were used to prepare the interconnect blocks for use by threading them. The reaction channel was 500 μm wide and 500 μm deep with a working volume of 240 μL connected to two inlet channels with similar dimensions by a T-junction (Figure 9). The reactor design was initially done by Filipe Carvalho for his doctoral thesis and is therefore not presented as a result of this chapter. However, the design was recreated by the author in SolidWorks 2015® (Dassault Systems, Vélizy-Villacoublay, France) and Adobe Illustrator (CC 2015.2, 19.2.0 Maidenhead, UK) for the purpose of the work presented in this thesis.
2.2.2. Fabrication of Micromixers

Micromixers were designed using Adobe Illustrator (CC 2015.2, 19.2.0 Maidenhead, UK) and a model of it was created using Solidworks 2015® (Dassault Systems, Vélizy-Villacoublay, France). The channels and cut-outs were fabricated out of 1.5 mm thick poly(methylmethacrylate) (PMMA) plates (Professional Plastics, Fullerton, California, USA) using a CO₂ laser marking head (Epilog Laser, Clevedon, UK). The layers were cleaned with water and thermo-compression bonded (50 min, 105 °C, under a pressure of 400 cNm which was achieved by applying a set torque on four screws that connected two aluminium plates).

2.2.3. Enzyme Production

2.2.3.1. Strains and Plasmids

Transketolase (WT-TK from E.coli BL21gold DE3 containing plasmid pQR791, Stratagene,
La Jolla, California, USA) and ω-transaminase (CV2025 from *E. coli* BL21gold DE3 containing plasmid pQR801) were produced in-house and stored at -80 °C in lysogeny broth (LB) broth containing 50% (v/v) glycerol.

### 2.2.3.2. Enzyme Expression

#### 2.2.3.2.1. Transketolase

Overnight cultures of *E. coli* BL21 were prepared in 10 g L\(^{-1}\) LB broth supplemented with 150 μg mL\(^{-1}\) ampicillin and 10 g L\(^{-1}\) glycerol. Cells were sub-cultured using 1% (v/v) inoculum in 2 L shaken flasks containing 500 mL of the same supplemented LB broth at 37 °C and 250 rpm until the bacterial growth had reached stationary phase, as confirmed by optical density at 600nm. Cells were harvested via centrifugation (10000 rpm, 20 min, 4 °C) (Beckman Coulter, Avanti J6-MI, Indianapolis, USA).

#### 2.2.3.2.2. Transaminase

Overnight cultures of *E. coli* BL21 were prepared in 10 g L\(^{-1}\) LB broth supplemented with 30 μg mL\(^{-1}\) kanamycin and 10 g L\(^{-1}\) glycerol. Cells were sub-cultured using 1% (v/v) inoculum in 2 L shaken flasks containing 500 mL of the same supplemented LB broth at 37 °C and 250 rpm. The transaminase production was induced with 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG, Calbiochem through Merck, Massachusetts, USA) when growing in early exponential phase (Optical Density 600 nm of 0.7, OD\(_{600}\) = 0.7) and temperature was reduced to 30 °C after induction until harvesting. Cells were harvested by centrifugation 5 h after induction (10000 rpm, 20 min, 4 °C) (Beckman Coulter, Avanti J6-MI, Indianapolis, USA). 15 min before harvesting pyridoxal-5’-phosphate (PLP) was added to a final concentration of 400 μM to improve enzyme stability during lysate preparation.
2.2.3.2.3. **Lysate Preparation**

The cell pellets were re-suspended in 50 mM Tris-HCl pH 7 and sonicated on ice using 10 on-off cycles for 10 s each at 100% (Soniprep 150 sonicator, Sanyo, Japan). The sonicated suspension was centrifuged at 5000 rpm at 4 ºC for 5 min. The lysates were stored at -20 ºC until use.

2.2.3.2.4. **Alternative Transaminase Production Method in Terrific Broth**

For transaminase experiments performed at pH 9, the cells were grown in 46.7 g L⁻¹ Terrific Broth, the growth was induced with 0.1 mM IPTG at OD 0.3 and shaken overnight at 200 rpm. PLP was not added before the harvesting of the cells. Harvest and storage conditions remained unchanged.

2.2.4. **Transketolase Activity Determination**

Reactions were carried out in 96 well microtiter plates containing 150 µL of a 100 mM HPA and 100 mM GA solution, by adding 150 µL of transketolase lysate solution (15 µL of TK lysate, 4.8 mM ThDP and 19.6 mM MgCl₂). Both solutions were prepared in 50 mM Tris-HCl buffer, pH 7. Transketolase solution was previously incubated at room temperature for 30 min. Final concentrations were 50 mM HP, 50 mM GA, 2.4 mM ThDP and 9.8 mM MgCl₂. Solutions were homogenised with pipette and left to react at room temperature. Using a sacrificial well approach, a reaction volume of 50 µL was removed at 1 min intervals for 4 min and quenched with 450 µL 0.1% (v/v) aqueous trifluoroacetic acid (TFA), centrifuged (5000 rpm, 5 min) and the supernatant analysed by HPLC. One transketolase unit (U) was defined as the amount of transketolase that catalysed the conversion of 1 mmol of substrate per minute in the aforementioned conditions.
2.2.5. Transaminase Activity Determination

Reactions were carried out in 96-well microtiter plates containing 180 µL of 10 mM MBA and 17 mM pyruvate by adding 20 µL of 1:10 diluted transaminase lysate previously incubated in 1 mM PLP. Both solutions were prepared in 50 mM phosphate buffer pH 7.4. Solutions were first warmed up individually to 32 °C, then homogenised with a pipette and left to react at 32 °C in a plate reader (FLUOstar Optima, BMG Labtech Ltd, Aylesbury, UK). The products of this reaction are alanine and acetophenone. The reactions were monitored for acetophenone formation at 280 nm for 2 min. One transaminase unit (U) was defined as the amount of transaminase that catalyses the conversion of 1 mmol of substrate per minute in the aforementioned conditions.

2.2.6. Cofactor Optimisation

Transaminase activity trials were performed as mentioned above, with varying concentrations of TK cofactors ThDP and MgCl₂, both together and individually to determine inhibition effects with 0, 2.4, 3.84, 4.8 and 6 mM ThDP and 9.8, 15.6, 19.6 and 24.5 mM MgCl₂.

2.2.7. Enzyme Reactions

2.2.7.1. Transketolase – Batch Reactions

Reactions were performed in 10 mL vials with 7 mL of reaction media and mixing was promoted by magnetic stirring. Substrate concentrations were 50 mM HPA and 50 mM GA and transketolase concentrations ranged from 0.63 to 2.85 U mL⁻¹. Transketolase cofactors concentrations were 2.4 mM ThDP and 9.8 mM MgCl₂. All solutions were prepared in 50 mM Tris-HCl buffer, pH 7. Prior to reaction, transketolase was incubated with the cofactors at room temperature for 30 min. 50 µL samples were removed at required time intervals and quenched with 450 µL 0.1% (v/v) aqueous trifluoroacetic acid (TFA), centrifuged (5000 rpm, 5 min) and the supernatant analysed by HPLC.
2.2.7.2. Transketolase - Continuous Flow Reactions

The setup included a two-syringe system such that substrate and enzyme were placed into separate syringes. In the syringe containing the substrates, the concentrations were: 100 mM HPA and 100 mM GA. In the other syringe transketolase concentrations ranging from 2.00 to 8.07 U mL\(^{-1}\), 4.8 mM ThDP and 19.6 mM MgCl\(_2\) were used. Solutions were pumped into separate inlets of the microfluidic reactor at the same flow rate (in the range of 2 to 120 \(\mu\)L min\(^{-1}\)) for at least 1.5 times the residence time before samples were taken. Samples were collected at the outlet of the reactor in pre-weighed sample vials containing 360 \(\mu\)l 0.1% TFA. The quenched reaction mixture was centrifuged and analysed by HPLC as above. Residence times were calculated by dividing the reactor volume (240 \(\mu\)L) by the flow rate.

2.2.7.3. Transaminase - Batch Reactions

Reactions were performed in 1.5 mL Eppendorf tubes containing initially 250 \(\mu\)L of a solution of the transaminase lysates incubated with 2 mM pyridoxal-5-phosphate (PLP). 125 \(\mu\)L of 40 mM MBA and 125 \(\mu\)L of 100 mM ERY were added. All the solutions were prepared in 50 mM Tris-HCl buffer, pH 7.5. The final concentrations were 25 mM ERY, 10 mM MBA, 1 mM PLP and TAm in the range of 1.90 and 5.24 U mL\(^{-1}\). Solutions were homogenised with pipette and allowed to react at room temperature. A volume of 40 \(\mu\)L was removed at the required time intervals, quenched with 360 \(\mu\)L 0.1% (v/v) aqueous trifluoroacetic acid (TFA), centrifuged (5000 rpm, 5 min) and the supernatant analysed by HPLC.

2.2.7.4. Continuous Transketolase and Transaminase Reactions

The setup comprised two reactors, carrying out the transketolase and transaminase reaction respectively, connected by Upchurch® connectors and fittings (P-221, Upchurch Scientific, through VWR, Pennsylvania, USA). Syringe pumps (KDS210, KD Scientific, Holliston, MA, USA) were connected to the reactors at different point in the fluidic path (Figure 10).
Transketolase reactions were carried out under identical conditions as previously described with an activity of 3.25 \text{ U mL}^{-1}. For the transaminase reaction, one syringe containing 40 mM MBA (pH 10, initial pH) and a second containing 27.0 \text{ U mL}^{-1} TAm lysate and 2 mM and PLP respectively at pH 9, were connected to the micromixer as shown in Figure 10 (final concentrations of 10 mM MBA and 10.8 \text{ U mL}^{-1} TAm were thus obtained). The flow rate for the TAm syringe was varied from 2 - 40 \text{ µL min}^{-1}. All other inlet flows were set at half the TAm flow rate. Samples were taken after three (mean) residence times, quenched with 0.1\% (v/v) TFA and analysed by HPLC as described above. Solutions were pumped at the same flow rate into separate inlets of the microfluidic reactor. Final concentrations in the reactor were 10 mM MBA and 5.2 \text{ U mL}^{-1} of TAm. The TAm reactors consisted of a PTFE coil (PTFE, ID 0.75 mm, VWR International Ltd, Lutterworth, UK) with length of 6.79 metres and a volume of 3 mL.

Flow rates were the same for all the pumps used in the setup and were varied from 1.25 to 6.25 \text{ µL min}^{-1} in order to achieve the desired residence times. Total flow rates in the transaminase reactor were in the range of 5 to 25 \text{ µL min}^{-1}. Samples were collected and analysed in HPLC.
after quenching with 0.1% (v/v) TFA. To compare batch and continuous reactions the residence
times were normalised according to Marques et al. (Marques et al., 2012) (See Section 2.3.1).

2.2.8. Analytical Methods

2.2.8.1. Protein Quantification

Bradford reagent (Sigma-Aldrich, Gillingham, UK) was used for total protein concentration
quantification with bovine serum albumin (BSA, Sigma-Aldrich, Gillingham, UK) as standard
(Bradford, 1976).

2.2.8.2. HPLC Detection Method

HPA and ERY were analysed directly using an Aminex HPX-87H column (300 mm x 7.8 mm;
Bio-Rad, Hemel Hempstead, UK) with 0.6 mL min-1 isocratic elution of 0.1% (v/v) TFA at 60
°C and detection at 210 nm. MBA and AP were quantified with an ACE 5 C18 RP column (150
mm x 4.6 mm, 5 µm particle size, Advanced Chromatography Technologies, Aberdeen, UK).
The mobile phase was comprised of 0.1% (v/v) TFA at 1.0 mL min-1 with a gradient of
acetonitrile from 15% to 72% over 9 min, followed by a 2 min equilibration and detection at
254 nm. ABT was derivatised by diluting the samples with 0.2 M borate buffer pH 8.8 and
adding an excess of 6-aminouinolyl-N-hydroxysuccinimidyl carbamate. ABT was quantified
using an ACE 5 C18 RP column (150 mm x 4.6 mm, 5 µm, Advanced Chromatography
Technologies, Aberdeen, UK) with a mobile phase comprised of 140 mM sodium acetate buffer
(adjusted to pH 5 using acetic acid) at 0.5 mL min-1 with a gradient of acetonitrile from 85%
to 100% over 10 min, followed by a column wash phase and re-equilibrium step at 1 mL min-
1 and detection at 254 nm. ABT for calibration purposes was synthesised according to (Ingram
et al., 2007).
2.3. Results and Discussion

The optimisation of the transketolase-transaminase cascade started before the beginning of this project. Previous work included the early stages of the optimisation of the transketolase reaction. This work was performed by Filipe Carvalho as part of his doctoral thesis at Instituto Superior Técnico Lisboa, part of the University of Lisbon. In section 2.3.1 the key results of this optimisation step are summarised due to their importance for the later optimisation steps undertaken by the author of this thesis.

2.3.1. Optimisation of Transketolase Reaction in Microfluidic Device

In previous studies regarding the production of ERY from HPA and GA 100% conversion of 50 mM GA and 50 mM HPA was achieved in 120 min of reaction time using a concentration of 2.28 mg.ml⁻¹ of total protein on the lysate (O'Sullivan et al., 2012). In the work undertaken by Filipe Carvalho, the transketolase concentrations in the microreactor were increased, while the substrate concentrations remained unchanged. In order to quantify the reaction progress, the increase of ERY and the decrease of HPA were determined via HPLC analysis. By increasing the transketolase input, a reduction of the reaction time to full conversion was achieved, showing a plateau after which a further excess of enzyme no longer reduced the reaction time.

For this reason, the minimum amount of transketolase that achieved a full conversion of 50 mM HPA/GA to 50 mM ERY in less than 10 min was chosen for all further transketolase experiments undertaken as part of this thesis. The determined amount was 3.25 U mL⁻¹ and presented as ‘Condition 5’ in Table 5 and Figure 11. Increasing the enzyme concentration further brought no additional benefits as the system was operating at its kinetic limitation. Any further enzyme addition would only increase the cost of the process.
Table 5: Transketolase reaction conditions and corresponding times of conversion

<table>
<thead>
<tr>
<th>Condition</th>
<th>Enzyme concentration (mmol min(^{-1}) mL(^{-1}))</th>
<th>Protein concentration (mg mL(^{-1}) in reactor)</th>
<th>Time for complete conversion (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.00</td>
<td>0.47</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>1.60</td>
<td>0.75</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>2.00</td>
<td>0.94</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>2.58</td>
<td>1.18</td>
<td>12</td>
</tr>
<tr>
<td>5</td>
<td>3.25</td>
<td>1.47</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>4.04</td>
<td>1.84</td>
<td>8</td>
</tr>
</tbody>
</table>

Figure 11: Transketolase reaction profile with increasing enzyme activity where A) corresponds to the 1\(^{st}\) condition, B) to the 2\(^{nd}\) condition, C) to the 3\(^{rd}\) condition, D) to the 4\(^{th}\) condition, E) to the 5\(^{th}\) condition and F) to the 6\(^{th}\) condition in Table 5. A conversion of 100% corresponds to 50 mM ERY.

As a part of this optimisation study, the cofactor concentration of the transketolase cofactors thiamine pyrophosphate (ThDP) and magnesium chloride was increased proportionally to the transketolase concentration.

For the purpose of the thesis the reaction was repeated using condition number 5 to verify the results. HPA and ERY were monitored via HPLC analysis, as the GA and the by-product carbon dioxide could not be monitored with the analytical methods employed for this study. Since the stoichiometry of the reaction for both substrates and products is at a 1:1 ratio, it can be assumed
that the GA decrease corresponds with the HPA decrease. The reaction profile can be found in Figure 12, showing full conversion (50 mM) after 10 min of reaction time.

![Figure 12: Transketolase reaction profile of the production ERY and consumption of HPA using an enzyme concentration of 3.25 U mL\(^{-1}\) (5\(^{th}\) condition according to the thesis of Filipe Carvalho)](image)

Filipe Carvalho’s research also showed that the reaction time and conversion between batch and microfluidic reactor setups were comparable with only minor differences within standard error. To compare the performance of batch and microfluidic reactor the normalised residence time (NRT) was calculated to reflect the enzyme activity (\(\sigma\)) according to Marques \textit{et al.} (Marques \textit{et al.}, 2012). For a batch reactor, \(\sigma\) is defined as:

\[
\sigma = \frac{E_t \cdot t}{V_s}
\]

Where \(E_t\) is the total amount of enzyme (U), \(t\) is the reaction time (min) and \(V_s\) is the reaction volume (mL). For the continuous reactor, \(\sigma\) is defined as:

\[
\sigma = \frac{E_t}{F}
\]

Where \(F\) corresponds to the flow rate (mL min\(^{-1}\)), \(\sigma\) has the U min mL\(^{-1}\).
2.3.2. Passive Micromixer Studies

The use of passive mixing structures in a channel can be an effective strategy for enhancing the mixing process in microfluidic environments. Two different passive mixing structures were tested in this work to determine whether they could benefit the presented reaction setup at the intended flow rates. The tested mixing structures were a staggered herringbone and a tesla structure (Figure 13). Both structures are efficient mixers when operating at flow rates larger than 250 µL min⁻¹ (Nguyen, 2011). In this present work the structures were tested for comparatively low flow rates to determine whether they could enhance the conversion in the microchannel. The reactors with the passive mixing structures were designed before the start of this project and manufactured by Marco Marques. The passive mixing structures were micromilled into the channel after the T-junction where the substrate and enzyme streams from the two separate inlets meet and form a reaction zone. According to Ristenpart et al. (Ristenpart et al., 2008) a reaction could be diffusion-limited due to the substrate diffusing faster than the enzyme because of its smaller size, compared to the enzyme.
To test whether the passive mixing structures could enhance the efficiency of the reaction, the transketolase reaction was performed using condition 5, as described in Table 5. The serpentine reactor of Figure 9 was used as a control experiment and the reaction volume of all reactors was adjusted to be equal by using tubing pieces (PTFE, 0.75 mm internal diameter) of varying length to compensate for any difference in volume. The total reactor volume was 240 μL for all reactors. The reaction was performed at a flow rate of 80 μL min$^{-1}$, which corresponded to a residence time of 3 min, roughly 60% of substrate conversion was previously observed. In order to be able to compare the conversions of the reactors the conversion for each reactor was normalised in ratio to the reactor with the highest conversion, the Tesla structured mixer, where 62% total conversion were achieved. The result is displayed in Figure 14.
Figure 14: Normalised conversion of HPA/GA to ERY through the transketolase reaction in the different reactors with mixing structures. TESLA - reactor containing tesla mixing structures, SHM - staggered herringbone mixer

The results showed that performing the reaction in microreactors with passive mixing structures under the presented conditions did not further improve or benefit the reaction in comparison to the unstructured serpentine reactor. For this reason, it was decided to avoid passive mixing structures for the other experiments performed to optimise this reaction cascade. A structure like the Tesla structure could even impact the reaction negatively, as the contractions in the channel could lead to biocatalyst build-up and clogging of the channel. It is possible that these passive mixing structures could benefit the reaction if the reactors were operated at higher flow rates, as has been shown by O’Sullivan et al. (O’Sullivan et al., 2012), where staggered herringbone microreactors were used to enhance mixing. The authors tested various flow rates ranging from 30 to 300 µL min\(^{-1}\) and shared the observation that no significant improvement of the conversion was achieved at lower flow rates, while higher flow rates produced favourable results. It is likely that the convection necessary to significantly increase the Reynolds number of the system does not occur at flow rates as low as the ones used in this study. At the presented flow rate, the Dean flow created by the turns of the serpentine reactor likely contributes more
to the mixing than any passive mixing created by the additional structures (Labeed and Fatoyinbo, 2014).

2.3.3. Cross-Reaction Studies of TK-TAm Reaction Components

Initial attempts at coupling the transketolase reaction with the transaminase reaction in flow did not result in product formation. For this reason, cross-reactivity between the components of the transketolase and transaminase reaction was investigated. Combinations of the transketolase reaction’s product (ERY) and the transaminase reaction’s co-substrate MBA were incubated at room temperature in different buffers (Tris and HEPES, 50 mM, pH 7) in order to determine whether there was a change in concentration during storage. Samples were taken daily for the first three days and again after six days and analysed via HPLC. Figure 15 shows the results of this investigation. Only a slight fluctuation of concentration was observed, consistent with small errors in sample preparation. Therefore, a spontaneous reaction between ERY and MBA could be ruled out.

![Figure 15: Cross-sensitivity study of ERY (50 mM) and MBA (Left: 25 mM, Right: 5 mM)](image-url)
The same experiment was repeated with the addition of transketolase in the reaction mixture. This was done in order to rule out a side reaction between transketolase when the two substrates of the transaminase reaction were present in the solution (Figure 16).

![Figure 16: Cross-sensitivity study of ERY (50 mM), MBA (Left: 25 mM, Right: 5 mM) and transketolase concentrations consistent with condition 1](image)

Again, no notable decrease of MBA was recorded for this experiment. Therefore, a cross reactivity between transketolase, ERY and MBA could be excluded.

### 2.3.4. Enzyme Stability Studies

Previous studies showed a reaction time for the transketolase-transaminase reaction cascade of as long as 100 h (Ingram et al., 2007) could be necessary. Since this indicates long periods of time during which the enzymes would be exposed to room temperature, the stability of the enzymes was tested (Figure 17 and Figure 18). For this purpose, samples of the transketolase and transaminase from freshly produced batches were taken and stored at three different conditions: at room temperature (RT), 4 °C and -20 °C, always dark conditions. Each day (TAm) or week (TK), a sample was used for an activity test as described in the materials and methods section. The results were normalised according to the initial sample used on the first day of the experiment.
Figure 17: Long-term storage stability of transketolase stored under different conditions, showing a desirable stability for the first 7 days of storage followed by a significant decrease of activity by day 14 for all samples except the frozen samples, which remained active.
Since the transaminase reaction was slower than the transketolase reaction and more of the enzyme would be necessary in order to achieve a swift conversion, the enzyme would most likely spend less time at room temperature than the transketolase would, as it would require frequent replenishing. Therefore, samples were taken daily to assess the storage stability at room temperature. Storing the enzyme at room temperature for four days (such as mimicking a 100 h reaction time) resulted in an activity decrease of 47%. Decreasing the reaction time of this cascade is therefore highly desirable.

2.3.5. Optimisation of the Transaminase Reaction in Batch

The optimisation of the transaminase reaction was first attempted in isolation from the transketolase reaction. For this purpose, ERY was purchased and used as a substrate for the reaction. The reaction was performed in batch and with an excess of ERY (25 mM) in
comparison to MBA (10 mM). In previous work, an excess of ERY was recommended to overcome the unfavourable equilibrium of the reaction, though much higher ratios of 1:10 were previously reported (Abdul Halim et al., 2013, Villegas-Torres et al., 2015). Varying concentrations of transaminase were used for the reaction, while the substrate concentrations remained unchanged, to determine whether a kinetic limitation could be reached. In order to reduce cost and labour of the trial, the batch reaction was performed in a 1 mL volume in an Eppendorf tube as a reactor. The maximal biocatalyst concentration possible in the reactor was 5.2 U mL⁻¹. This TAm concentration corresponds to the use of the undiluted lysate from an average batch obtained from the biocatalyst production, when the lysate makes up 40% of the total reaction volume (Figure 19). Therefore, higher concentrations were not tested. The decrease of ERY and the increase of AP were tracked via HPLC analysis for a 20 h period, during which a conversion of 80% was achieved for the highest transaminase concentration. ABT was not quantified in this study, since the synthesis of the reference material had not been undertaken at that point in the optimisation process. Still, this trial was used to determine an approximate reaction time for the second step of the cascade. The trial also showed that the reaction comes to an end after 10 h of reaction time, which was chosen as a point of reference for the reactor used in the coupling of the reactions. This was vital for decisions regarding the reactor configuration for the continuous flow synthesis. When the reaction is performed as part of the cascade, it is vital that HPA and GA have been fully converted to ERY in the TK reaction step (Rios-Solis et al., 2011)(Abdul Halim et al., 2013), due to the transaminase’s higher affinity towards glycolaldehyde as a substrate.
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Figure 19: Transaminase reaction profile of the production ABT in batch using commercially available substrates ERY and MBA. The consumption of MBA (A) and the production of AP (B) are shown.

The use of commercially available ERY has the upside that no other components of the transketolase reaction can be present in the sample, since the ERY purchased from Sigma Aldrich was produced through a fermentation process. The downside of this fact is that the ERY sample contains unknown contaminants from the alternative production process that might have a detrimental effect on the transaminase activity. The ERY used in these experiments was a hygroscopic solution with a water content of 14.2% and showed some amounts of impurities. These impurities were not present in the ERY produced via the TK reaction. This could be seen as a further advantage of producing an intermediate through biocatalysis. During multiple transaminase batch trials, multiple different batches of commercially available ERY were used, leading to different conversion times in the transaminase reaction. Therefore, the results obtained from these trials can only be used as guidelines.

2.3.5.1. Transketolase Cofactor Optimisation for Transaminase Reaction

When the coupling of the transketolase and transaminase reaction was attempted in a sequential batch trial, no production of AP or ABT was observed. This led to a detailed inhibition study.
of all the components in the cascade reaction. The results showed that the activity of transaminase was severely reduced by an increase in the transketolase cofactor concentration of thiamine pyrophosphate (Figure 20). Therefore, the transketolase reaction was re-optimised upon the discovery of this fact. It was found that during the initial optimisation of the transketolase reaction, the cofactor concentrations had been increased in accordance with the transketolase activity increase. This led to cofactor concentrations of 6 mM thiamine pyrophosphate and 24.5 mM magnesium chloride being used in the coupled reaction. When activity trials were performed using these concentrations in solution with transaminase, an activity reduction of up to 30% was observed. The transketolase reaction was re-optimised to a low cofactor concentration of 1.2 mM ThDP and 4.8 mM MgCl₂ which still served to achieve a full conversion in the transketolase reaction in 10 min.
Figure 20: Cofactor inhibition studies for transaminase using various concentrations of MgCl₂ (A), ThDP (B) and a combination of the two (C). Activity determined through photometric detection of acetophenone increase at 280 nm, experiments performed in batch.
2.3.5.2. Microfluidic Cascade of Transketolase and Transaminase

Following the adjustment of the transketolase cofactor concentration it was possible to show positive results for the coupled transketolase-transaminase reaction in flow. For this purpose, the transketolase reaction was performed in the reactor introduced in Figure 9, the outlet of the reactor was coupled with a micromixer (Figure 21) that had one primary inlet for the TK reaction mixture and one primary inlet for the MBA. The TK reaction mixture is thereby used to dilute the MBA to avoid an initial inhibition of the TAm enzyme. The TAm was then added through a secondary inlet further down in the reactor. The micromixer had a channel width of 500 μm and a channel depth of 450 μm and a total internal volume of ~200 μL. The micromixer outlet was attached to a coil reactor. The reactors were all coupled together according to the reactor schematic in Figure 21.

Figure 21: Exploded view of micromixer laser-fabricated from poly(methylmethacrylate) (PMMA) and showing the connector bars
At a TAm concentration of 1.7 U mL$^{-1}$, a conversion of 80% was achieved after 6 h of reaction time (Figure 22). While 6 h of reaction time represent a great improvement from previously reported 25 to 100 h, further improvement was necessary.

![Graph showing reaction profile for the TAm reaction in continuous flow under non-optimised conditions](image)

**Figure 22**: Reaction profile for the TAm reaction in continuous flow under non-optimised conditions

### 2.3.5.3. Optimisation of Transaminase Production and pH Adjustment

One of the bottlenecks in the optimisation of the transketolase-transaminase cascade was the production of transaminase in sufficient amounts needed for experiments at multiple residence times. A switch to a different growth medium (terrific broth) and an alternative production protocol (lower IPTG concentration and induction at a lower OD) was attempted and yielded large improvements (Table 6). Furthermore, it was decided that the transaminase reaction should be performed at pH 9, since the transaminase used for these experiments was shown to have an activity maximum at pH 9 by Schell et al. (Schell et al., 2009). The two protocols are summarised in Table 6.
Table 6: Comparison of two different protocols for transaminase production

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Protocol 1</th>
<th>Protocol 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>10 g L(^{-1}) LB Broth with 10 g L(^{-1}) glycerol</td>
<td>47.6 g L(^{-1}) Terrific Broth</td>
</tr>
<tr>
<td>Induction OD</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td>Growth time after induction</td>
<td>5 h</td>
<td>12 h</td>
</tr>
<tr>
<td>IPTG Concentration</td>
<td>1 mM</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>PLP addition before harvest</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Extraction buffer pH</td>
<td>pH 7</td>
<td>pH 9</td>
</tr>
<tr>
<td>Pellet weight per 400 mL media</td>
<td>1.6 g</td>
<td>4.5 g</td>
</tr>
<tr>
<td>Final activity after extraction</td>
<td>15 U mL(^{-1})</td>
<td>27 U mL(^{-1})</td>
</tr>
</tbody>
</table>

Switching to Protocol 2 resulted in an 80% increase in activity and a tripling of biomass yield for the transaminase production.

2.3.6. Optimised Microfluidic Cascade of the Transketolase and Transaminase Reaction

Having optimised the reaction pH, transaminase activity and cofactor concentrations for the transketolase reaction, the enzymatic reactions were coupled in a microfluidic flow again. Mean residence times between 30 min and 10 h were tested for the cascade. 50 mM HPA and GA were converted into 50 mM ERY in the first reactor at a total flow rate ranging from 2-40 µl min\(^{-1}\), the resulting product stream was then diluted by an input of 50 mM MBA at half the flow rate of the TK reaction, and then by a TAm input of the same flow rate, resulting in a total flow rate in the final reactor from 5 to 100 µL min\(^{-1}\). The cascade was run for 2.5-3 residence times before samples were taken to be analysed via HPLC. The flow rates were chosen due to reactor length constraints. Higher flow rates would require larger reactor volumes in order to accommodate sufficient residence times, which would be more expensive; as such a reaction would require a larger amount of enzyme and substrate input. On the other hand, lower flow rates than the ones used in this study were deemed unreliable given the combination of syringe pumps with plastic tubing and syringes. As it was, a total system flow rate of 5 µL min\(^{-1}\) required an input of 1 µL min\(^{-1}\) of the MBA substrate stream, which was the lowest reliable flow rate for
the KD scientific pumps. Through the reaction compartmentalisation into the different reactors, it was possible to regulate the pH reliably for each reaction, so that the TK reaction pH was 7, and through the input of the MBA stream at pH 10 and the TAm lysate, the TAm reaction pH was 9. The transketolase reaction was completed in less than 10 min with a volumetric activity of 3.25 U mL⁻¹, and the transaminase reaction was done with a volumetric activity of 10.8 U mL⁻¹. Figure 23 shows the resulting NRT profiles of the reaction in batch and flow for comparison, with full conversion achieved after only 2 h of reaction time. This translates into a ten-fold reduction of reaction time compared to the earliest results achieved in batch with commercially available ERY before optimisation steps for the reaction system were undertaken.

![Figure 23: Comparison of coupled TK-TAm cascades in sequential batch (left) and microfluidic reaction cascade (right) for the residence times up to 2 h. To compare batch and continuous reactions, the residence times were normalised according to (Marques et al., 2012)](image)

The corresponding changes in concentration for each substrate and product in batch and continuous flow are summarised in Figure 24.
These improvements are due to an optimisation of the transketolase cofactor concentration, an increased transaminase activity due to the optimisation of the production protocol and an adjustment of the reaction pH for the TA\textsubscript{Am} reaction. The final ABT yield obtained in this microfluidic reactor cascade is comparable with, or higher than, previous transketolase-transaminase-catalysed reactions in flow setups.

To compare, a final ABT concentration of 5 mM was achieved in a capillary packed bed reactor with purified immobilised enzymes by Abdul Halim \textit{et al.} (Abdul Halim \textit{et al.}, 2013). Using a substrate concentration of 60 mM HPA and GA and 6 mM MBA (corresponding to a 10:1 substrate ratio of ERY and MBA), the final concentration of ABT (83\% conversion) was reached in 20 min. In contrast, the here-presented system only required a substrate ratio of 2:1, significantly reducing the ERY waste. Matosevic \textit{et al.} (Matosevic \textit{et al.}, 2011a) used TK and
TAm immobilised on AB-NTA derivatised micro capillaries and produced 0.3 mM of ABT in 40 min using 10 mM of MBA, HPA and GA (such as an equimolar ratio of ERY MBA). ABT was not directly measured in this study. ABT was previously produced in one-pot batch systems using the TK-TAm enzymatic reactions, yet full conversion was not shown. Villegas-Torres et al. (Villegas-Torres et al., 2015) showed a conversion limitation at 2.5 mM ABT after 50 h using L-serine as a cosubstrate instead of MBA. The reaction was limited by side reaction, which illustrates the disadvantage of a one-pot reaction due to the lack of overlap of ideal reaction conditions that suit both enzymes. A full conversion of the TAm reaction step was only achieved by Halim et al. (Halim et al. 2014), but the reaction was performed with purified ERY instead of a transketolase reaction. Additionally, the TAm reaction step required a secondary reaction to shift the reaction equilibrium, or the implementation of in situ product removal strategies (ISPR). Overall, the here-presented cascade shows a high throughput coupled with an easy to implement setup and demonstrates the advantages of a microfluidic setup for continuous production.

2.4. Conclusion and Suggestions for Future Work

Through optimisation steps it was possible to reduce the production time for the production of 2-amino-1,3,4-butanetriol from over 20 h to only 2 h in flow and to achieve full conversion that was previously not possible. This was achieved using free enzymes for both steps and required improvements towards the transaminase activity, cofactor concentrations and pH adjustment. With the improvements towards the transaminase activity, cofactor concentrations and pH adjustment it was possible to make this reaction cascade a viable route for continuous production. However, the system could still be further improved. Examples for this are the use of purified enzymes, which could increase the volumetric enzyme activity and thereby increase the reaction speed. The TK cofactor (ThDP) that causes inhibition in the TAm reaction step could be immobilised in the reactor used for the TK reaction (Benítez-Mateos et al., 2018).
Immobilising the cofactors for both reactions, rather than incubating them with the enzymes might also increase enzyme activity, as it was shown that dimeric and tetrameric enzymes were more stable, but less active when incubated with their cofactors (Börner et al., 2017). The use of a multi-input microreactor (Lawrence et al., 2013) or side-entry reactor (Gruber et al., 2017d) mimicking fed-batch operation is an attractive approach to reach higher productivities while avoiding enzyme deactivation due to substrate inhibition. Enzyme recycling steps could reduce enzyme wastage, which could be achieved by introducing a microfiltration unit after each reactor (O'Sullivan et al., 2012). By adding optical sensors and control strategies to maintain optimal reaction conditions, better reaction control of the individual reactors could be achieved. The incorporation of in situ product removal (ISPR) strategies as previously shown by Halim et al. (Halim et al., 2014) could further shift the unfavourable equilibrium of the TAm reaction towards the product side. This could also be achieved by adding a liquid-liquid extraction unit operation, which would separate AP and ABT into the organic and aqueous phase respectively and aid product recovery. Furthermore, the replacement of MBA with the cheaper and more water-soluble amine donor isopropyl amine could also speed up the TAm reaction rates due to the lower inhibitory nature of IPA, however, the by-product acetone might lead to inhibition of the enzyme in higher concentrations (Rios-Solis et al., 2011).
3. Development and Proof of Concept of Microfluidic Diels-Alder Reaction and Transketolase Reaction Cascade

3.1. Introduction

Chemo-enzymatic reactions are an area of synthesis for which the application of microreactors has thus far been underutilised. There have been very few examples of a chemical reaction coupled with an enzymatic reaction, or vice versa, in microreactors (Luckarift et al., 2007). The coupling of chemical and enzymatic reactions is of high interest for pharmaceutical development. Such reactions present an opportunity for the versatile synthesis of complex chiral synthons. However, the interrupted execution of the sequential batch reactions, interspersed with product recovery and purification steps, is associated with an increased process time and a reduction in product yield. This reduction is due to loss of product in the purification steps. The use of a continuous flow reaction cascade would reduce both the material loss and the reaction time.

The proof of concept for a novel asymmetric synthesis of chiral compounds will be attempted in this chapter, using a Diels-Alder reaction followed by a transketolase-catalysed reaction. These reactions were previously explored individually or in a step-wise synthesis, interrupted by purification steps.

The specific Diels-Alder – transketolase reaction cascade presented in this chapter can be used for the synthesis of 3,4-dimethylcyclohex-3-ene-2’-keto-1’,3’-propanediols, which contains two chiral centres. The model reaction system that is the focus of this chapter is presented in Figure 25. This synthesis has not been presented in a microfluidic cascade or continuous production without intermediate purification steps.
Figure 25: Diels-Alder – transketolase reaction scheme for the two-step chemoenzymatic synthesis of 1-(3,4-dimethyl-3-cyclohexen-1-yl)-1,3-dihydroxypropan-2-one (DCDHP). ThDP: thiamine diphosphate

The 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde (CCA) enantiomers can be synthesised from two simple, relatively affordable, non-chiral starting compounds, acrolein and 2,3-dimethyl-1,3-butadiene, via asymmetric Diels-Alder reactions using enantio-complementary organocatalysts. The reaction mechanism of this reaction can be seen in Figure 26.

Figure 26: Diels-Alder reaction mechanism showing the ring-closure between a conjugated diene and a substituted alkene to form a substituted cyclohexene

The Diels-Alder reaction, first published in 1928 (Diels and Alder, 1928), has become an often-used and versatile synthesis methodology for the production of numerous complex molecules in a series of organic reaction sequences (Nicolaou et al., 2002). Despite its long history in research, as well as industrial applications (Funel and Abele, 2013), the Diels-Alder reaction has seldom been used in combination with an aqueous environment in reaction. It has been reported that the use of an aqueous environment can lead to an acceleration of the reaction (Rideout and Breslow, 1980) and the development of catalytic enantioselective Diels-Alder reactions (Corey, 2002, Ahrendt et al., 2000, Aragonès et al., 2016).
However, this area of development is a prerequisite in order to interface this chemical reaction with biocatalytic reactions (Wohlgemuth, 2007). Various catalytic Diels-Alder reactions are described for the synthesis of racemic 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde (Cavill et al., 2003, Chen et al., 2004, Taarning and Madsen, 2008, Abbott et al., 2002, Nakashima and Yamamoto, 2005, Stevens and Pagenkopf, 2010) and for asymmetric Diels-Alder reactions of 2,3-dimethyl-1,3-butadiene and acrolein (Chu et al., 2006, Benaglia et al., 2002, Furuta et al., 1989, Barroso et al., 2008). None of the named reactions have previously been performed in microfluidic flow and most catalysts and reaction conditions presented are not suitably compatible with the subsequent enzymatic reaction. The catalysts and solvents that were potentially suitable for a subsequent transketolase reaction were screened in this work.

Two organocatalysts (5R)- and (5S)-5-((1H-indol-3-yl)methyl)-2,2,3-trimethylimidazolidin-4-one hydro-chloride (Figure 27) were produced by our project partners at Sigma Aldrich in order to synthesise the racemic mixture as well as the two separate enantiomers (R and S) of 3,4-dimethyl-3-cyclohexene-1-carboxaldehydes. The enantiomers were analysed for enantiomeric purity by chiral GC, showing a 90% enantiomeric excess each.

![Figure 27: Structure of chiral catalyst to selectively produce the (R) and (S) enantiomers of CCA](image)

While these catalysts were crucial for the production of the individual enantiomers, the reactions they catalysed were found to be too slow for a viable flow system, with reaction times of >3 days. However, this approach made it possible to determine the enantioselectivity of transketolase towards one of the intermediate enantiomers (Figure 28).
An optimisation of the Diels-Alder reaction was necessary in order to achieve a high throughput and faster conversion. This required the screening of other Diels-Alder catalysts.

The carboxaldehydes formed in the organocatalytic Diels-Alder reaction then enabled the investigation of the subsequent reaction. The transketolase-catalysed two-carbon chain extension was performed using hydroxypyruvate as a donor, which renders the reaction irreversible due to the release of carbon dioxide as a side product.

The enzymatic reaction using transketolase has been described for the cascading enzymatic reaction system (Chapter 2). For both of these reactions it is of advantage to work in a microfluidic system, given that the components used for the reaction are expensive and synthesis at this scale could make the optimisation process more cost effective. For this reaction, a one-point mutant of the wild type transketolase was used in which the aspartic acid (D) in position 469 was replaced with a threonine (T) (D469T). This mutation allows the transketolase to accept cyclic substrates like CCA where the wild type is restricted to smaller linear substrates, such as glycolaldehyde. The mutant was selected based on Daniel Pais’ (Pais, 2013) and Lydia Coward’s (Coward, 2012) work where the wild type, the D469T mutant and a three-point mutant were screened for this reaction. The packed-bed reactor was linked to a coil reactor using a four-way valve that allowed the supply of co-substrate, hydroxypyruvate and transketolase.
3.2. Materials and Methods

Unless stated otherwise, all chemicals and reagents were purchased from Sigma-Aldrich (Gillingham, UK) and were used without further purification.

3.2.1. Strain and Plasmid

The D469T transketolase mutant was previously cloned into an expression vector (pQR791) and kindly provided by Prof. John Ward’s group, Department of Biochemical Engineering, UCL. Additional information about D469T (pQR791, includes a His-tag, XL10-gold E. coli cells, Stratagene, La Jolla, California, USA) can be found in Payongsri et al. (Payongsri et al., 2012).

3.2.2. Enzyme Production

The cells were grown in LB media consisting of 10 g L⁻¹ LB medium powder, 10 g L⁻¹ glycerol and 150 μg mL⁻¹ of ampicillin. This concentration was the same for both, agar plates and liquid cultures. The cells were produced in 2 L shake flasks filled with 500 mL media and shaken for an average of 9 h after inoculation at 37 °C and 250 rpm. After the growth, cells were harvested using a centrifuge (10000 rpm, 20 min, 4 °C) (Beckman Coulter, Avanti J6-MI, Indianapolis, USA). The cells were re-suspended in 50 mM TrisHCl buffer pH 7.0 at a ratio of 4 mL per gram of wet biomass pellet. The cells were lysed through sonication using a Soniprep 150 sonicator (Sanyo, Japan) on ice for 10 cycles of 10 s sonication followed by 10 s pause. A cell-free extract was obtained by clarifying the lysate from cell debris through centrifugation at 4 °C for 5 min at 5000 rpm (Eppendorf 5415R Refrigerated Benchtop Microcentrifuge, Eppendorf, Hamburg, Germany).

For experiments in which the enzyme was purified, the cells were re-suspended in a binding buffer (20 mM sodium phosphate, pH 7.4, 500 mM sodium chloride, 20 mM imidazole)
compatible with the Ni-NTA column used for purification at a ratio of 4 mL per gram of wet biomass pellet.

3.2.3. **His<sub>6</sub> Tag Purification**

After column equilibration, the clarified lysate was applied to a Ni-NTA column (HisTrap HP, 5 mL, GE Healthcare, Buckinghamshire, UK) with binding buffer and washed with 5 column volumes of binding buffer after which the enzyme was eluted with elution buffer (Table 7). The purified fractions were tested for activity using the D469T activity assay as described below after a buffer exchange. The column was stripped and recharged with 0.5 mL of a 0.1 M nickel sulfate solution after five purification runs.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Sodium phosphate</th>
<th>Sodium chloride</th>
<th>Imidazole</th>
<th>EDTA (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding</td>
<td>20</td>
<td>500</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Elution</td>
<td>20</td>
<td>500</td>
<td>500</td>
<td>-</td>
</tr>
<tr>
<td>Stripping</td>
<td>20</td>
<td>500</td>
<td>-</td>
<td>50</td>
</tr>
</tbody>
</table>

3.2.4. **Buffer Exchange**

For the buffer exchange from elution buffer to 50 mM Tris buffer pH 7.0, the purified lysate was filled into dialysis tubing (BID-020-010D BioDesignDialysis Tubing™ (D011), Fisher Scientific made by BioDesign Inc, New York, USA). The ends were tied into knots and enforced with cable ties. The tubing was submerged in 200x the dialysate volume of 50 mM Tris buffer pH 7.0. The container was left in the refrigerator overnight to ensure successful dialysis.

3.2.5. **D469T Activity Protocol**

The transketolase activity assay was adapted from Pais (Pais, 2013). As with the activity assay for the wild-type transketolase, the D469T mutant is incubated with its cofactor solution at
concentrations 2.4 mM for ThDP and 9.6 mM MgCl₂ for 30 min before the substrate stock solutions are added. The substrates 3-formylbenzoic acid and Li-β-hydroxypyruvate are added as stock solutions to a final concentration of 50 mM each. Samples are taken at the time-points of 1, 2 and 3 min and mixed with 0.1% trifluoroacetic acid to stop the reaction and determine initial conversion rates. The components of the assay are detected in the samples using the HPLC analysis described below. The method file for each compound can be found in the appendix. The activity trial was performed in batch format in a 1.5 mL Eppendorf tube. One transketolase unit (U) was defined as the amount of transketolase which catalyses the conversion of 1 mmol of substrate per minute at pH 7.0 and 20 °C.

3.2.6. HPLC Analysis

HPA was analysed directly using an Aminex HPX-87H column (300 mm x 7.8 mm; Bio-Rad, Hertfordshire UK) with 0.6 mL min⁻¹ isocratic elution of 0.1% (v/v) TFA at 60 °C and detection at 210 nm. 3-formylbenzoic acid was quantified with an ACE 5 C18 RP column (150 mm x 4.6 mm, 5 µm particle size, Advanced Chromatography Technologies, Aberdeen, UK). The mobile phase was comprised of 0.1% (v/v) TFA at 1.0 mL min⁻¹ with a gradient of acetonitrile from 15% to 72% over 9 min, followed by a 2 min equilibration and detection at 254 nm.

3.2.7. GC Analysis

CCA was quantified using a ThermoScientific Trace 1300 FID gas chromatograph with an Rxi® 5 Sil MS column (30 metres, 0.25 mm ID, 0.5 µm df, Sigma Aldrich, Gillingham, UK). The samples were analysed in split-flow mode with a temperature gradient from 40 °C to 140 °C in 25 min, followed by an increase from 140 °C to 180 °C over 3 min. CCA is detected at a retention time of 18.5 min (Figure 29).
3.2.8. UHPLC-MS Analysis

The samples generated through batch reactions of the R and S enantiomers of CCA with HPA and transketolase were dried under air flow and re-suspended in a water:methanol:acetonitrile 1:2:1 mixture. The sample was run over an Ascentis Express RP-Amide, 2.1 x 50 mm, 2.7 µm column (Sigma Aldrich, Gillingham, UK) with a 0.1% ammonium acetate gradient with methanol starting from 5% methanol and ending with 95% methanol after 7 min at a flow rate of 0.6 ml min⁻¹. The mass spectrometer used was a Bruker qTOF-microTOF QII run with positive ion polarity at a full scan from 50 to 1000 Da and a spectra rate of 5 Hz.

3.2.9. Assay for DCDHP Standard Solutions

50 µL of sample are mixed with 20 µL tetrazolium red solution (0.2% 2,3,5-triphenyltetrazolium chloride in methanol) and 10 µL, 3 M NaOH (aq) are added and mixed by FLUOstar Optima plate reader (BMG Labtech Ltd, Aylesbury, UK). This was followed by immediate measurement at OD₄₈₅nm. Standard solutions from 0.0625 mM to 3.5 mM of DCDHP
were prepared. The calibration curve proved to be linear from 0.0625 to 1.75 mM DCDHP (Figure 30). DCDHP was prepared in-house at Sigma Aldrich, Steinheim, Germany.

![Figure 30: DCDHP calibration curve achieved through colorimetric assay showing a linear increase of absorbance from 0.0625 to 1.75 mM DCDHP](image)

$$y = 0.1785x + 0.0215$$

$$R^2 = 0.9997$$

3.2.10. Assay for Samples Containing HPA

50 µL of sample are transferred to a 96-well plate and 10 mg MP-carbonate resin is added and left to incubate for 3 h. 50 µL of TrisHCl buffer was added to each microwell and pipette-mixed. This solution (50 µL) was then transferred to a fresh microwell plate. 20 µL tetrazolium red solution (0.2% 2,3,5-triphenyltetrazolium chloride in methanol) and 10 µL, 3 M NaOH (aq) are added and mixed by FLUOstar Optima plate reader (BMG Labtech Ltd, Aylesbury, UK). This was followed by immediate measurement at OD_{485nm}.

3.2.11. Thin-Layer Chromatography

For the thin-layer chromatography, a Si_{60} F_{254} aluminium-supported TLC plate was used. The samples were applied by filling 5 µL capillaries and spotting them onto the plate. A 1:1 mixture
of ethyl acetate and n-hexane served as a mobile phase. The plate was developed using a 10% phosphomolybdic acid stain in ethanol and a hot plate. The \( R_f \) value for CCA was determined to be 0.92 and the \( R_f \) value for DCDHP was 0.52 under these conditions.

### 3.2.12. Synthesis of R-CCA and S-CCA

The S and R enantiomers of CCA were synthesised in batch reactions and kindly provided by our project partners at Sigma Aldrich, Member of Merck Group. For the S enantiomer, 0.113 mol of 2,3-dimethyl-1,3-butadiene and 0.05 mol of acrolein were stirred in 18 mL of water and 32 mL of methanol with 1 g of (5R)-5-(1H-indol-3-ylmethyl)-2,2,3,4-trimethyl-4-imidazolidinone monohydrochloride as a chiral catalyst for 3 days, resulting in a 16.6% yield after purification. For the R enantiomer, 0.283 mol of 2,3-dimethyl-1,3-butadiene and 0.067 mol of acrolein were stirred in 18 mL of water and 32 mL of methanol with 1.2 g of (5S)-5-(1H-indol-3-ylmethyl)-2,2,3,4-trimethyl-4-imidazolidinone monohydrochloride as a chiral catalyst for 3 days, resulting in a 12.9% yield. Both enantiomers were purified by extracting them with a 50 mL trifluoroacetic acid:water:chloroform 1:1:2 mixture for 10 min under stirring. Ether was added and the aqueous phase was extracted thrice, dried over magnesium sulfate and filtered. The solvent was evaporated and the residue was further purified via column chromatography.

### 3.2.13. Diels-Alder Aluminium Chloride Packed-Bed Reactor Assembly

A blank column kit with column dimensions of 4.6 mm x 0.63 cm x 15 cm (ID x OD x L) was purchased from Sigma Aldrich (Gillingham, UK). The column was cut down to a length of 3.5 cm, achieving a volume of 1 mL. Aluminium chloride immobilised on silica gel (particle size: 40-63 μm) was purchased (Sigma Aldrich, Gillingham, UK) and filled into the empty column under nitrogen atmosphere. 0.52 g of immobilised catalyst was used to fill the column completely. This resulted in a reactor volume of 490 μL. The reactor was then washed with acetonitrile for 10 column volumes before the Diels-Alder reaction was run.
3.2.14. Diels-Alder Reaction in Flow

200 mM of acrolein and 200 mM of 2,3-dimethylbutadiene in acetonitrile (GC grade, Sigma Aldrich, Gillingham, UK) were pumped through the packed bed reactor at a flow rate of 4 µL min⁻¹, resulting in a residence time of 2 h.

3.2.15. Diels-Alder Reaction using Purified Enantiomers in Batch

A 40 mM solution of 90% enantiomerically-pure (R)-CCA and 40 mM HPA was stirred in a 10 mL glass vessel overnight in a 1:1 reaction mixture with TK D469T at a final activity of 2.6 U mL⁻¹. The mixture was pre-incubated with 2.4 mM thiamine diphosphate and 9.8 mM magnesium chloride in 50 mM Tris-HCl buffer pH 7.0.

3.2.16. Transketolase Flow Reaction

The transketolase was diluted to an activity of 4.92 U mL⁻¹ with a 50 mM Tris-HCl buffer (pH 7.0). The enzyme was then incubated with 2.4 mM thiamine diphosphate and 9.8 mM magnesium chloride. The transketolase reaction was performed with 20 mM CCA, 10 mM HPA and a final enzymatic activity of 3.94 U mL⁻¹. Taking into account the effect of 10% acetonitrile on transketolase, which resulted in a total deactivation of 27%, the final transketolase activity per mL reactor volume was 2.86 U mL⁻¹.

3.2.17. Diels-Alder – Transketolase Reaction Cascade Assembly

The aluminium chloride packed-bed reactor was operated at a flow rate of 4 µL min⁻¹ within three days of being assembled. The Diels-Alder product stream was then diluted with 100 mM HPA in 50 mM TrisHCl buffer pH 7.0 at 4 µL min⁻¹ and a flow of transketolase prepared as previously described at a flow rate of 32 µL min⁻¹.
3.2.18. Thin-Layer Chromatography Mass Spectrometry (TLC-MS) Analysis

A CAMAG, TLC-MS Interface was used to extract spots from TLC plates in order to analyse individual components from the DA-TK reaction system that had been previously separated on SI60 F254 aluminium-supported TLC plates. In order to accurately locate the spots, a second silica plate was spotted and developed in parallel to the plate from which the spots would be extracted. The reference plate was then stained with phosphomolybdic acid as described and the location of the spots optically approximated on the unstained plate intended for extraction. A schematic for the TLC-MS interface is shown in Figure 31. An HPLC pump flushed the extractor head with solvent, thereby washing the analyte off the TLC plate, through a filter and into a sample vial. The sample was then analysed via UHPLC-MS.

Figure 31: Schematic of TLC-MS interface used for extraction of analytes from TLC plate. The different coloured spots indicate different components on the TLC plate
3.3. Results and Discussion

3.3.1. Determination of Transketolase Substrate Selectivity

In order to determine the substrate selectivity of the D469T transketolase mutant towards the R- or S-enantiomer of CCA, the transketolase reaction was performed with the selectively-produced enantiomers of each chirality in a batch reaction. The selectively-produced R- or S-enantiomer stocks only contained <10% of the respective other enantiomer. The reaction was stirred overnight and then centrifuged to remove the enzyme. The remaining water was evaporated under air flow and the sample re-suspended in a water:methanol:acetonitrile 1:2:1 mixture of 40 µL and analysed via mass spectrometry. A clear mass peak was detected at 199 m/z, corresponding with the product mass Figure 32.

![Figure 32: MS analysis of TK reaction with (R)-CCA enantiomer and HPA performed after an overnight reaction. The analysis confirms the presence of DCDHP in the reaction mixture with a peak at 199 m/z](image)

This mass peak was prominent in the reaction performed with the R enantiomer and very minor in the reaction performed with the S-enantiomer, which contained <10% of the R-enantiomer.
TLC analysis further confirmed the formation of DCDHP when using the R-enantiomer Figure 33.

Figure 33: TLC analyses of TK reaction of S-CCA and R-CCA confirming TK selectivity towards R-enantiomer. LEFT: The TLC plate shows the CCA S-enantiomer in the first lane, the product of the reaction with the S-enantiomer in the second lane, the unreacted R-enantiomer in the third lane and the reaction mixture after reaction with the R-enantiomer in the fourth lane. There is a clear DCDHP spot in the fourth lane, confirming the presence of the product after the TK-catalysed reaction of HPA and R-CCA. RIGHT: The TLC plate shows HPA in the first lane as a reference, the racemic mixture of the R-CCA and S-CCA in the second lane. The product of the TK-catalysed reaction with HPA and the racemic mixture is shown in the third lane, with a weak DCDHP spot. The product of the TK-catalysed reaction with the R and S enantiomer are shown in the fourth and fifth lane, respectively. The DCDHP spot is most prominent in the fourth lane, indicating the TK selectivity towards the R-enantiomer.

3.3.2. Selection of Suitable Diels-Alder Catalyst

Four catalysts, zinc chloride, scandium triflate, aluminium chloride and (5S)-5-(1H-indol-3-ylmethyl)-2,2,3-trimethyl-4-imidazolidinone monohydrochloride, were screened for the production of CCA in a microreactor. All catalysts were tested in batch using four different solvents which have previously been used for Diels-Alder reactions, or have been tested to be compatible with transketolase, with no deactivation of transketolase when used at a
concentration of up to 10%. The tested solvents were acetonitrile, methanol, ethanol and isopropanol. However, samples containing methanol or water could not be analysed due to incompatibility with the GC column, only TLC analysis could be used to confirm the presence of CCA in solutions containing water or methanol. However, none of the TLC plates showed the presence of CCA in any of the water- or methanol-containing samples. Table 8 shows the conversions achieved after 2 h reaction time at room temperature with substrate concentrations of 200 mM.

Table 8: Summary of solvent/catalyst combinations and product concentration after 2 h reaction time at 20 °C

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Catalyst</th>
<th>Soluble?</th>
<th>CCA concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>ZnCl₂</td>
<td>Y</td>
<td>4.8</td>
</tr>
<tr>
<td>Methanol</td>
<td>ZnCl₂</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>Ethanol</td>
<td>ZnCl₂</td>
<td>Y</td>
<td>0.6</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>ZnCl₂</td>
<td>N</td>
<td>1.6</td>
</tr>
<tr>
<td>Methanol</td>
<td>Chiral catalyst</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>Chiral catalyst</td>
<td>Y</td>
<td>1.5</td>
</tr>
<tr>
<td>Methanol</td>
<td>AlCl₃</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>Ethanol</td>
<td>AlCl₃</td>
<td>Y</td>
<td>0.1</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>AlCl₃</td>
<td>N</td>
<td>0.2</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>AlCl₃</td>
<td>N</td>
<td>37.5</td>
</tr>
<tr>
<td>95% Acetonitrile + 5% MeOH</td>
<td>AlCl₃</td>
<td>N</td>
<td>-</td>
</tr>
<tr>
<td>90% Acetonitrile + 10% MeOH</td>
<td>AlCl₃</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>95% Acetonitrile + 5% EtOH</td>
<td>AlCl₃</td>
<td>N</td>
<td>10.6</td>
</tr>
<tr>
<td>90% Acetonitrile + 10% EtOH</td>
<td>AlCl₃</td>
<td>N</td>
<td>3.9</td>
</tr>
<tr>
<td>80% Acetonitrile + 20% EtOH</td>
<td>AlCl₃</td>
<td>Y</td>
<td>0.8</td>
</tr>
<tr>
<td>95% Acetonitrile + 5% iPrOH</td>
<td>AlCl₃</td>
<td>N</td>
<td>12.8</td>
</tr>
<tr>
<td>90% Acetonitrile + 10% H₂O</td>
<td>AlCl₃</td>
<td>Y</td>
<td>-</td>
</tr>
<tr>
<td>70% Acetonitrile + 30% H₂O</td>
<td>AlCl₃</td>
<td>Y</td>
<td>-</td>
</tr>
</tbody>
</table>

The combination of acetonitrile and aluminium chloride showed the highest potential for a full conversion, but aluminium chloride is not soluble in acetonitrile. Therefore, an immobilised catalyst approach was attempted. For this purpose, a blank HPLC column kit (Sigma Aldrich, Gillingham, UK) was used to create a packed-bed reactor with an empty volume of 1 mL and a packed volume of 490 µL (Figure 34). These volumes were determined by measuring the
residence time of acetonitrile pumped at 10 µl min in the reactor. This was done three times to determine the volume. The column length was chosen to be the shortest possible length that would still allow the assembly with the connectors as shown in Figure 34.

![Figure 34: Packed-bed reactor with aluminium chloride immobilised on silica gel](image)

The column was then closed and flushed with acetonitrile for 3 h at a flow rate of 50 µL min⁻¹. During this time, a washout of loosely-bound catalyst took place, in the form of a bright red-purple outlet stream with white precipitate (Figure 35) which subsided after 3 h. The outlet stream ran clear after this time period, though additional colour-less washout of precipitate over time cannot be excluded.

![Figure 35: Initial aluminium chloride wash-out after column packing](image)

After an initial washing of the column with 10 column volumes of acetonitrile, a 200 mM 2,3-dimethyl-1,3-butadiene and 200 mM acrolein mixture in acetonitrile was pumped through the column at a flow rate of 4 µL min⁻¹, which equated to a residence time of 2 h. This led to a full conversion to 200 mM CCA, which was confirmed via GC-FID analysis and unlike with the
commercially available CCA, there were no significant other peaks visible in the chromatogram. A 2 h experiment was repeated for three consecutive days to determine the catalyst stability in the column. During that time no decline in CCA production was observed. After 1 week of storage in acetonitrile, the resulting yield was only one third of the initial CCA production (see Figure 36). At this point the column was opened and repacked.

![Figure 36: Column activity decline after one week of use, showing a steep decline in product concentration at day seven](image)

It is worth noting that the flow rate of 4 µL min⁻¹ means that the reactor was operated at a low flow-rate to accommodate the reaction time of the transketolase reaction. Full conversion was also achieved when the reactor was operated at a flow rate of 10 µL min⁻¹. Flow rates higher than that were not tested since they would not match the transketolase reaction.

### 3.3.3. Effect of Solvent on the Activity of Transketolase

Initial studies on solvent compatibility were done using 10% 2-isopropanol and 10% methanol, both of which led to clearly observable conversion from CCA to DCDHP as could be seen through visualisation TLC plates (see Figure 37).
The activity of the D469T TK mutant was determined according to the standard activity protocol presented in Section 3.2.5. In order to determine the influence of various solvents on the activity of the enzyme, 10% (v/v) were added to the reaction. The initial activity of the reaction without solvent was compared to the initial activity with 10% solvent. The results in Figure 38 show that, while there is a distinct decrease in activity, the enzyme retained sufficient activity to be used for a cascade reaction. A 27% decrease in activity was detected at 10 v/v% of acetonitrile in solution.
3.3.4. **Reaction Time Determination with Transketolase Batch Reactions**

In order to determine the residence time required for achieving a full conversion in the biocatalytic reaction step of the cascade, a batch reaction was performed. This was done using 100 µL of the 60 mM Diels-Alder reaction performed in the packed bed reactor, 100 µL of 30 mM of hydroxypyruvate and 800 µL of transketolase, resulting in a total TK activity in the reactor of 2.86 U mL$^{-1}$. Samples were taken every 15 min and the reaction was quenched via a 1:10 dilution of the sample with 0.1% trifluoroacetic acid (TFA). The samples were analysed for HPA depletion using HPLC, which resulted in a time-course reaction profile for the transketolase reaction. The HPA depletion curve suggests that the reaction achieves full conversion after only 45 min (Figure 39).
3.3.5. Microfluidic Diels-Alder – Transketolase Cascade Reaction

In order to couple the two reactions, the packed-bed reactor was connected to a coil reactor using a four-way valve (PEEK 1/8 0.050 CROSS (HP), Gilson Scientific LTD, Dunstable, UK) (Figure 40). A 200 mM mixture of 2,3-dimethyl-1,3-butadiene and acrolein in acetonitrile was pumped through the packed-bed reactor at a flow rate 4 µL min⁻¹. The resulting flow was then diluted with 4 µL min⁻¹ of 100 mM hydroxypyruvate, as racemic CCA is formed in the Diels-Alder reaction catalysed by aluminium chloride. The third inlet was used for a 32 µL min⁻¹ input of 2.86 U min⁻¹ transketolase. For the reaction coupling in a cascading flow system, a coil reactor that could accommodate a reaction time of 75 min was chosen (3 mL volume, 6.8 m, PTFE, ID 0.75 mm, VWR International Ltd, Lutterworth, UK) due to the low substrate concentrations in the batch reaction. A schematic reactor setup can be seen in Figure 40.

The production of DCDHP was confirmed through TLC analysis of the product stream, which showed a clear spot at the same Rᵣ value as the DCDHP standard (Figure 41). Furthermore, in order to achieve a quantitative result, a colorimetric assay was used to determine the DCDHP...
content of the sample. The assay indicated a DCDHP concentration of 3.5 mM, which corresponds with the solubility limit of the compound.

**Figure 40:** Microfluidic reactor setup of the cascade reaction as a schematic (left) and fully assembled (right)

**Figure 41:** TLC analysis depicting clear DCDHP production during the microfluidic Diels-Alder – transketolase reaction cascade. DCDHP standard confirms $R_f$ value of the product
3.4. Conclusion and Suggestions for Future Work

In this chapter, the proof of concept for the microfluidic cascade of a Diels-Alder reaction, followed by a transketolase reaction was shown. The use of microreactors in sequence made it possible to immediately convert the unstable intermediate of the Diels-Alder reaction (CCA) and thereby avoid intermediate recovery steps. In order to achieve a fast reaction and a high throughput, multiple catalysts and solvents were tested to find the ideal conditions for the Diels-Alder reaction, resulting in full conversion of a 200 mM equimolar reaction after only 50 min. This was achieved using a packed-bed reactor containing aluminium chloride immobilised on silica gel. The enantioselectivity of the transketolase was determined to be specific to the (R)-enantiomer of the Diels-Alder reaction product. Multiple catalysts and solvents were tested. The formation of the desired final product (DCDHP) in batch was demonstrated by mass spectrometry and TLC analysis and a reaction profile of the transketolase reaction was generated in batch. Finally, TLC analysis confirmed the DCDHP production in a microfluidic cascade in only 200 min at a concentration of at least 3.5 mM. It was shown that the transketolase enzyme used can withstand 10% v/v of acetonitrile. This is crucial in order to establish a cascading system.

While a clear proof of concept of the DA-TK cascade as a novel synthesis route in a microfluidic chemoenzymatic cascade has been shown through the iterations shown in this chapter, there is still room for improvement:

- A higher temperature (37 °C to 50 °C) could be used to decrease the reaction time of the transketolase reaction. This could allow the Diels-Alder packed-bed reactor to be operated at optimal conditions, rather than at low flow rates to accommodate the TK reaction rates, which would increase throughput.

- In order to increase conversion, other TK mutants could be tested to achieve a higher yield. In order for this to be possible the mutants would have to be at least as solvent-
resistant as the D469T mutant. Ideally, it would also be temperature-resistant in order to be able to further optimise the reaction.

- *In situ* product removal in the form of resin could be used in order to drive the reaction further towards the product side by overcoming a possible product inhibition that might be due to a solubility limit of DCDHP.

Furthermore, HPA is used as ketol-donor for the transketolase reaction in this system as well as in the bi-enzymatic reaction system. For the transketolase reaction of the chemoenzymatic cascade reaction, HPA is the only analyte that can easily be quantified using standard HPLC methods, rather than having to rely on a combination of methods to verify both quantity and quality. With this substrate the only side product is CO$_2$, which is an advantageous side product that does not require additional purification steps and drives the reaction irreversibly towards the product side of the equation. However, since the reaction occurs in a closed system, the CO$_2$ cannot escape the reaction mixture, leaving it as dissolved carbon dioxide in solution. Dissolved CO$_2$ can increase the pH in the liquid system as has been shown for the TK-TAm reaction cascade. For this reason, the implementation of pH sensors could be interesting for both monitoring and the confirmation of reaction progress.
4. Development of pH and CO\textsubscript{2} Sensors for Integration into Microfluidic Reactors

4.1. Introduction

The production of carbon dioxide, as a by-product in the transketolase reaction of both the transketolase-transaminase and Diels-Alder- transketolase reaction cascades, means that a pH change can occur due to the equilibrium of carbon dioxide and water, which leads to the generation of hydrogen carbonate.

\[ \text{CO}_2 (g) \rightleftharpoons \text{CO}_2 (aq) \]
\[ \text{CO}_2 (aq) + \text{H}_2\text{O} (l) \rightleftharpoons \text{H}_2\text{CO}_3 (aq) \]
\[ \text{H}_2\text{CO}_3 (aq) \rightleftharpoons \text{H}^+ (aq) + \text{HCO}_3^- (aq) \]
\[ \text{HCO}_3^- (aq) \rightleftharpoons \text{H}^+ + \text{CO}_3^{2-} (aq) \]

An increase of hydrogen carbonate in the reaction mixture can lead to a pH increase. The extent of that increase depends on many parameters, such as substrate and buffer concentration, as well as the initial pH and temperature. In order to be able to monitor this change two possible sensor options were explored: pH sensors in order to directly measure the pH increase in the system and carbon dioxide sensors to measure carbon dioxide directly, the molarity of which can be used to determine the pH shift mathematically. For this purpose, pH and carbon dioxide sensing system were miniaturised and integrated into microfluidic reactors during a secondment at Graz University of Technology. The results of the sensor development will be discussed in this chapter following an introduction to pH and carbon dioxide sensors, where sensing principles and previous applications are discussed.

4.1.1. pH Sensors

The pH is an important parameter for many biotechnological processes. It is crucial for the activity of many enzymes, the stability of products, substrates and cofactors and the chemical state (protonated, deprotonated) of components in the reaction. For microfluidic systems, optical sensors are often used for process monitoring since they allow online measurements
through the reactor material. Many of these sensors are based on the photoluminescence of the sensor dye used. Photoluminescence is a term that summarises the phenomena of fluorescence, phosphorescence and delayed fluorescence. These phenomena are often used for analytics due to their selectivity and specificity. They occur due to the emission of a photon from either an excited singlet or triplet state and can be most easily explained using the Jablonski diagram (Figure 42). Fluorescence occurs through the emission of a photon from an excited singlet state, while phosphorescence is the result of a photon being emitted from an excited triplet. The triplet state is a result of intersystem crossing from a singlet state. The difference between the two is their lifetime. While fluorescence has a lifetime of $10^{-10}$ to $10^{-8}$ s, phosphorescent lifetime is much longer with $10^{-6}$ to ~1 second. The use of these phenomena for analytics is popular because they are selective in most cases and highly sensitive, as emission spectra are specific to the dyes used in the sensor. Therefore, the chance of interference of other substrates in the reaction mixture is unlikely (Valeur, 2001).

![Jablonski Diagram](image)

Figure 42: Perrin-Jablonski diagram depicting the process of excitation and fluorescent and phosphorescent emission. A molecule can return from its excited state (S1 or S2) to its ground state (S0) by emitting absorbed energy through fluorescence, phosphorescence, photon emission (hv), internal conversion (IC) or intersystem crossing (ISC). Delayed fluorescence occurs when a molecule first crosses into the excited triplet state (T1), then returns to S1 through reversed intersystem crossing (rISC) before returning to S0 through fluorescence. Diagram from Grüber, 2014 (Gruber, 2014), adapted from Valeur, 2001 (Valeur, 2001)
The sensors usually consist of a dye embedded in a permeable polymer, often silicone-based, that allows them to interact with the surrounding environment. To avoid leaching of the sensor dye, the dye is either covalently bound to the polymer, or entrapped by crosslinked polymers. Crosslinking forms a mesh that retains the dye, but allows the analyte to permeate into the polymer matrix (Aigner et al., 2013). The following Table 9 and Table 10 show a list of often-used pH-sensitive dyes for the monitoring of pH in microfluidic systems, benchtop reactions, or larger systems. The dyes are grouped by absorbance- and fluorescence-based dyes. The dyes that are used for their absorbance-based pH indicator qualities are commercially available, while the fluorescence-based dyes used for these purposes are usually specifically modified for the application.

Table 9: Popular absorbance-based pH-sensitive dyes. These dyes are commercially available from Sigma Aldrich, Member of Merck group. The ranges are cited from their material safety data sheets

<table>
<thead>
<tr>
<th>Dye</th>
<th>pH range</th>
</tr>
</thead>
<tbody>
<tr>
<td>m-cresol purple</td>
<td>1.2 - 2.8, 7.4 - 9.0</td>
</tr>
<tr>
<td>Bromocresol purple</td>
<td>5.2 - 6.8</td>
</tr>
<tr>
<td>Phenol red</td>
<td>6.8 - 8.2</td>
</tr>
<tr>
<td>Cresol red</td>
<td>1.8 - 2.0, 7.0 - 8.8</td>
</tr>
<tr>
<td>Thymol blue</td>
<td>1.2 - 2.8, 8.0 - 9.2</td>
</tr>
</tbody>
</table>

Table 10: Popular fluorescence-based pH sensitive dyes

<table>
<thead>
<tr>
<th>Dye</th>
<th>pH range</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diketopyrrolopyrrolys</td>
<td>5 - 12*</td>
<td>(Aigner et al., 2013)</td>
</tr>
<tr>
<td>8-Hydroxy-1,3-pyrene-tris- sulphoic acid</td>
<td>2 - 14</td>
<td>(Mills and Chang, 1994)</td>
</tr>
<tr>
<td>Nile Red</td>
<td>4 - 9</td>
<td>(Ali et al., 2011)</td>
</tr>
<tr>
<td>Seminaphtho-rhodafluor</td>
<td>7 - 8*</td>
<td>(Borisov et al., 2011)</td>
</tr>
<tr>
<td>Seminaphtho-fluorescein</td>
<td>6.2 - 7.8 *</td>
<td>(Zhou et al., 2009)</td>
</tr>
<tr>
<td>Fluorescein isothiocyanate</td>
<td>5.5 - 7.5</td>
<td>(Lanz et al., 1997)</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>5 - 7.5</td>
<td></td>
</tr>
<tr>
<td>Aza-BODIPY dyes</td>
<td>2-12</td>
<td>Strobl et al. (2015)</td>
</tr>
</tbody>
</table>

*depending on functional groups

For optical sensors, the pH is determined through changes in the absorbance or fluorescence when the sensing dye is protonated or deprotonated. Fluorescence intensity and dual lifetime
referencing (DLR) are commonly used as utilised principles for pH sensing. For the detection of pH, the indicator dye is lyophilised and covalently linked to or embedded in a hydrophilic polymer. The sensitivity of a single sensing dye is limited to an operational range of approximately three pH units, but the use of multiple pH dyes exhibiting the same spectral properties yet different pKa values could be used to produce a broad-range sensor or sensor array that covers larger pH-ranges, as shown by Strobl et al. (Strobl et al., 2015) who produced dyes to cover the pH range from pH 2 to pH 12 (Figure 43).

The calibration curves of sensing dyes as the ones described here are typically sigmoidal and can be described with a Boltzmann-fit. A multi-point calibration of at least 5 points achieved by buffers at different pH values is recommended for a reliable calibration curve. Boltzmann-fits for calibration curves are usually defined as follows:

\[ y = \frac{A_1 - A_2}{1 + e^{(x-x_0)/dx}} \]

where \( A_2 \) is the value for low fluorescence, \( A_1 \) is the value for high fluorescence, \( x_0 \) is the point of inflection (which refers to pKa’, also known as the apparent pKa) and \( dx \) is the slope at the point of inflection. The dyes described in Figure 43 are excitable with red light and emit in the
NIR spectral regions. They offer the advantage of avoiding readout noise from wavelength overlap with inherently UV active reaction components, such as enzymes. These dyes were chosen for this work for their suitable pH range, photostability, low cross-sensitivity to ionic strength, suitable excitation and emission wavelength and their compatibility with the host polymer. While pH sensors of various types have been previously integrated into microfluidic reactors, the number of publications on pH measurements at a microfluidic scale is small.

Jezierski et al. (Jezierski et al., 2013) integrated a fluorescent pH sensor system for online monitoring of a free-flow electrophoresis reactor in order to observe the pH gradient within. Funfak et al. (Funfak et al., 2009) showed optical pH monitoring with a flow-through fluorimeter using polymer particles doped with HPTS dye to monitor the pH during cell cultivation in a droplet-based flow system. Brigo et al. (Brigo et al., 2008) presented 300-400 µm tentagel resin beads with immobilised covalently-bound pH-sensitive azo-dye. These polyethylene glycol beads, attached to cross-linked polystyrene, were used in a PDMS on glass reactor. The measurement of the pH was achieved using a micro-photometer consisting of a confocal microscope, coupled to a diode-array spectrophotometer with an optical fibre.

A high throughput optical sensor array was developed for the online monitoring of the pH of a cell culture by Wu et al., in which the light transmission efficiency through various thicknesses of PDMS layers was studied (Wu et al., 2009).

Muñoz-Berbel et al. (Muñoz-Berbel et al., 2013) used dissolved indicator dye in a PDMS reactor with a monolithically integrated filter with microchannels for size exclusion to monitor a cell culture’s OD (at various wavelengths) and the pH by measuring the absorption of phenol red. Del-Ben et al. (Del Ben et al., 2016) used pH sensors to monitor the metabolism of circulating tumour cells (CTCs) to detect such cells in compartmentalised single-cell droplets on a microfluidic platform. Tahirbegi et al. (Tahirbegi et al., 2017) used pH sensors in combination with oxygen sensors to monitor the metabolism of algae in order to detect pesticides in the algae’s environment. Very few examples for continuous pH monitoring of
enzymatic reactions at a microfluidic scale are known. Ehgartner et al. showed the potential for monitoring both the pH and oxygen simultaneously using sensor beads (Ehgartner et al., 2016a).

### 4.1.2. Sensor Principles of pH Sensors Used for Transketolase Monitoring

The sensors used for this work are based on the principle of Dual Lifetime Referencing (DLR). With this detection method the overall signal of a reference dye, this is inert to the analyte and an indicator dye, which reacts to the analyte, is detected. The overall signal changes in the presence of the analyte in a reproducible manner that can be detected using a phase shift fluorimeter. The reference dye often serves as a way to produce a detectable signal, because the presence of the analyte often quenches the fluorescence of the indicator dye (Boniello et al., 2012). This concept is visualised in Figure 44:

![Figure 44: Dual Lifetime Referencing](image)

Figure 44: Dual Lifetime Referencing, the overall signal changes with the analyte concentration due to a change in emission behaviour in the indicator dye in ratio with the reference dye which is not sensitive to changes in the analyte concentration (Von Bultzingslöwen et al., 2002)

The reference dye used in this project was Egyptian Blue, which is an often-used and extremely stable pigment, resistant to photo-bleaching (Borisov et al., 2011).

### 4.1.3. Carbon Dioxide (CO₂) Sensors

Measuring carbon dioxide is a highly important topic as the carbon dioxide concentration is a crucial parameter in medicine (Mills et al., 1997), biotechnology (Weigl et al., 1994, Harms et al., 2002), food packaging (Von Bültzingslöwen et al., 2002) and oceanography (Doney et al., 2014).
Carbon dioxide is also a crucial parameter in fermentation processes and sometimes a by-product in enzymatic reactions such as that of a transketolase-catalysed reaction with hydroxypyruvate as a substrate (Woodley et al., 1996). The sensors that are used for detecting carbon dioxide are based on pH-sensitive dyes integrated into a polymer matrix alongside a base to form an ion pair with. The carbon dioxide concentration is based on a pH change in the sensor upon contact with carbon dioxide. The sensors can only be used in humid or aqueous environments because water molecules are necessary for the pH sensitivity, as can be seen through the following equilibrium

$$CO_2 + H_2O + Q^+Ind^- \rightleftharpoons Q^+HCO_3^- + HInd$$

where Q’ is the quaternary ammonium ion and Ind is the indicator dye, respectively.

Popular pH-sensitive dyes for absorbance include m-cresol purple (Mills et al., 1992), phenol red (Wolfbeis, 2005), cresol red and thymol blue (Mills and Chang, 1994). However, using fluorescence is usually preferable over measuring absorbance, due to increased detection limits and limited interference from surrounding light sources or components in the reaction mixture. Only very few publications have reported successful systems for the monitoring of carbon dioxide at a microfluidic scale. The CO₂ sensors most suitable for miniaturisation operate on the principle of a pH change in the sensor upon contact with CO₂. The pH change is measured via the optical change in the indicator dye. These sensors are usually referred to as dry-concept or solid-state sensors as described by Mills, or ‘Mills-type sensors’ (Mills et al., 1992). A Mills-type sensor consists of a hydrophilic polymer matrix and a quaternary ammonium ion as a base, which forms an ion pair with the pH-sensitive indicator dye. The pH sensitive layer is usually covered by a lipophilic protective layer to prevent interference from protons and other ions, which reduce the stability of the sensor by permanently protonating the dye.

The main challenge with the integration and use of carbon dioxide sensors is their limited long-term stability. The equilibrium between the quaternary ammonium base ion that is usually used
in excess for its buffering capacity within this system and the indicator dye is fragile and heavily influenced by acidic chemicals from the aqueous phase. Permanent protonation through non-analyte interaction severely limits the long-term stability of the sensors to only a few days and only if stored properly. Fritzsche et al. (Fritzsche et al., 2016) have taken steps towards improving the stability, but this has only been achieved in fibre-type sensors. The changes have yet to be adapted to a microfluidic scale. In a Mills-type sensor adaptation, Zilberman et al. (Zilberman et al., 2014) used cresol red and pyranine in combination with tetraoctylammonium hydroxide to create a sensing matrix. Calvo-Lopez et al. (Calvo-López et al., 2016) developed a credit-card-sized microsystem for the measurement of CO₂ during wine and beer fermentation, which has a linear range from 255 mg·L⁻¹ to 10,000 mg·L⁻¹ and a detection limit of 83 mg·L⁻¹. A gas diffusion module was used to transfer the carbon dioxide into a pH-sensitive acceptor solution, which was monitored at 607 nm. Sell et al. (Sell et al., 2013) documented the diffusion of carbon dioxide in water and brine by using fluorescence quenching of fluorescein. Liu et al. (Liu and Mathies, 2009) determined the solubility of carbon dioxide in water and brine with a confocal Raman spectroscope.

Since both cascading reactions, discussed in Chapter 2 and 3, feature a transketolase step in which HPA is used as a ketol-donor that results in CO₂ as a side product, integrating CO₂ sensors would close the mass balance of the reactions.

However, to the best of the author’s knowledge, no enzymatic reaction has been monitored with carbon dioxide sensors at a microfluidic scale yet and there have been no carbon dioxide sensor spots integrated into microfluidic reactors that were capable of monitoring small changes in dissolved carbon dioxide concentration.
4.1.4. Challenges of Adapting Optical Sensors for Microfluidic Systems

There is a clear need to adapt the existing technology for optical sensing towards the application and integration into microfluidic reactors. The main challenges to attain this objective can be summarised into the following key points:

a. Brightness: A main challenge for adapting any optical sensor technology into a microfluidic setup is the need to maintain sufficient brightness in the sensor read-out. This affects both the reliability of the read-out and the sensitivity, especially in sensor technology based on quenching. There is often a trade-off between intensity and sensitivity.

b. Homogeneity: While each sensor should always be calibrated individually, it is important to achieve some homogeneity in the sensor fabrication method. A similar brightness and sensitivity among sensors not only makes data processing easier, but also ensures reliability in the read-out. Furthermore, if all sensors are similar, time-consuming data processing is not strictly necessary in order for the user to be able to draw first conclusions on experimental outcomes from the read-out alone.

c. Stability: The sensors need to show stability against photo-bleaching and fouling. While some drifting is expected over time, the sensors have to be stable enough to suit their application. Sensors should not drift or bleach (for example through loss of sensitivity) during an experiment.

d. Specificity (carbon dioxide specific): To obtain a reliable read-out it is important to ensure the measured carbon dioxide concentration is a result of the carbon dioxide present in the solution or air. This is a challenge when the reaction takes place in a polymer vessel and the sensor is placed on top of a polymer. The polymer can act as a ‘carbon dioxide sink’ meaning it may store carbon dioxide, the presence of which may then be detected by the sensor and result in a false readout. In order to avoid this, the sensor must be shielded from other sources of carbon dioxide.
e. Chemical stability (carbon dioxide specific): A major challenge of all optical carbon dioxide sensor technology is the sensor stability. Due to the chemical equilibrium in the sensor, acidic components can compromise the sensor’s sensitivity (sensor poisoning). A protective layer is therefore needed to protect the main sensing layer from poisoning. However, the introduction of an additional layer must not restrict the carbon dioxide from reaching the sensor. Any additional layer is also likely to increase the sensor response time.

These challenges will be addressed in the Materials and Methods section where the fabrication methods relating to these challenges are presented, followed by a Results and Discussion section that showed the results of the development efforts to address these challenges and finally a Conclusion where further steps towards the remaining points that could not be addressed are summarised and advice is given on how they could be addressed in future projects.
4.2. Materials and Methods

4.2.1. Production of pH Sensor ‘Cocktails’

The pH sensor mixture, commonly referred to as ‘cocktail’ (Schutting et al., 2013), consisted of 10% HydroMed D4 (Advansource Biomaterials, Massachusetts, USA), 0.1% pH dye (Dye Number 3, (Strobl et al., 2015)) and 10% Egyptian Blue particles (CaCuSi₄O₁₀), produced at the Graz University of Technology (TUG) according to Berke et al. (Berke, 2007), dissolved in tetrahydrofuran (THF). If a crosslinking agent (MPAX, Bayer, Leverkusen, Germany) was used for the cocktail, it was prepared with dry materials under nitrogen atmosphere in a glovebox.

4.2.2. Production of the Carbon Dioxide Sensor ‘Cocktails’

The carbon dioxide sensor reference layer consisted of 6% polyethylene naphthalate (PEN) (w/w) and 6% Reference particles (w/w) (Egyptian Blue: Polystyrol particles in a ratio of 3:1) in a 1:1 mixture of hexafluoropropanol and chloroform. The indicator layer was composed of 5% ethylcellulose (w/w) and 0.05% Indicator Dye (w/w) (di-OH-di-F-azaBODIPY) in ethanol: toluene, 4:6. For generations 4 and 5, 0.1% of the indicator dye was used. The protective layer consisted of 5% Hyflon AD 60 perfluorodecaline (ACBR, Karlsruhe, Germany) which was previously extracted with NaHCO₃ to eliminate acidic residues in the solvent. The dyes were produced at TUG according to Jokic et al., (Jokic et al., 2012). The carbon dioxide sensors of generation 1 and 2 were knife-coated and glued into Microfluidic Chip-Shop flow-chamber reactors using Elastosil E4 silicone glue (Wacker Chemie AG, Munich, Germany). This was done by applying a thin layer of glue to the underside of the support foil with a spatula and letting the glue air-dry at room temperature.
4.2.3. Microdispenser-Assisted Integration of Sensor Spots into Reactors

The controlling unit was a Vermes Microdispensing MDC 3200+ and the dispensing unit was a Vermes microdispensing MDV 3200A-HS-UF, respectively. The CNC controller used was a Triple BEAST, high performance microstep driver, three-axis 55 V, 5 A (Controller) (Programmed via LinuxCNC). The software used to operate the dispenser is a LabVIEW routine, programmed in-house by Philip Sulzer from TUG. The tappet rod used was the Vermes TTF 20. The nozzle inserts that were used were the Vermes N11-200 for pH sensors, N11-70 for indicator dye in carbon dioxide sensors, the N11-400 for reference layer of carbon dioxide sensors and pH sensors and the N13-50 for indicator dye in carbon dioxide sensors. The pH sensors and generations 3-5 of the carbon dioxide sensors were integrated into the reactors using this microdispenser setup. The rising time used for dispensing pH sensors was 0.3 ms, the opening time was 0.2 ms and the fall time 0.12 ms using 40% lift. These times also applied for the use of the reference layer of the carbon dioxide sensors.

The pH sensors were produced by repeatedly spraying one spot to increase the brightness by creating a thicker spot. The carbon dioxide sensors are spotted in a grid pattern to cover more area and allow the read-out from a reactor with a higher sensor chamber with an optical fibre (See Figure 45). This grid type pattern was later expanded on for the other layers (Generation Three).

Figure 45: Grid pattern used to spray the sensor layers for carbon dioxide sensors. This pattern allowed an even application of the dyes over a wider surface area that was compatible with the optical fibre diameter needed to read out the sensors
4.2.4. Measurement Setup for pH Sensors

For the read-out of the pH sensors in the reactor, a 4-channel phase-shift fluorimeter (FireStingO2, Pyro Science GmbH, Aachen, Germany) was used. 1 m optical fibres (Pyro Science GmbH, Aachen, Germany) were fixed above the spots using polymer cubes with 1 mm holes that fit the fibres. The cubes were taped into place so that the fibre surface was in contact with the lid of the reactor above the spot. Tris-HCl buffer solutions with pH values ranging from pH 5-8 were used to calibrate the sensors.

4.2.5. Reactor Fabrication and Bonding for pH Sensing in Microreactors

The reactors used for the experiments were laser-cut from 1.5 mm thick PMMA sheets (Professional Plastics, Norfolk, UK) using a CO₂ Laser (Epilog Laser, Clevedon, UK). The reactors were bonded using a Collin platen press type S (Dr. COLLIN GmbH, Ebersberg, Germany) at 110 °C and a pressure of 5 bar. The reactors spent a total of 50 min at the set temperature of 110 °C in 10 min intervals in order to achieve full bonding. The press was self-adjusting to keep a constant pressure, so the bonding had to be performed in short intervals in order to ensure the melting of the plastic did not affect the integrity of the channels.

4.2.6. Measurement Setup for Carbon Dioxide Sensors

The carbon dioxide sensors were measured in a flow chamber reactor (Product number: 12-0902-0172-02, Microfluidic ChipShop, Jena, Germany) in gas phase only. The gases were 100% N₂, 5.06% CO₂ in N₂ and 100% CO₂, which were provided by Linde, Austria. The gases were humidified by flushing them through a bubble trap filled with silica gel and dampened with distilled water. The sensors were read out using a 4-channel phase-shift fluorimeter (FireStingO2, Pyro Science GmbH, Jena, Germany) and a 30 cm long fibre for generations 1 and 2 and a 1 m fibre for generations 3-5. Unless stated otherwise the sensors were read out using LED intensity set to 60% of the equipment capability. The reactors were sealed using a double-sided adhesive foil into which the outline of the chamber has been cut.
4.2.7. **Statistics**

The sensor read-out was set to occur once per second for 10 ms at the amplitude of 400 mV and a modulation frequency of 2 kHz. Each sensor was read out continuously. In this chapter, the continuous readout was either presented in full, or, when data points were presented, they were the average of 20 s of readout, meaning an average of 20 successive data points.
4.3. Results and Discussion

4.3.1. pH Sensors

4.3.1.1. Fibre-Type pH Sensors

Dyes number 1 and 3 were used for the pH sensor work of this chapter as published by Strobl et al. (Strobl et al., 2015). In order to determine the ideal dye for the application in the PMMA material that was chosen as reactor material, the dye’s stability and response in the sensor material had to be tested. For this reason, it was decided to produce fibre-type sensors, meaning the sensors were applied to foil (Figure 46) that were then fixed to the ends of optical fibres (Pyro Science GmbH, Aachen, Germany) (Figure 47 and Figure 48). The aim was to test the long-term stability and response time in that format as it was expected to give an approximation of the dye’s behaviour in the reactor. The sensor cocktails were prepared under nitrogen atmosphere as the crosslinking agent and catalyst are highly reactive to the presence of oxygen. The foils were produced by knife-coating the sensor cocktail onto a PET foil (125 μm in thickness) using a 1 mm knife, resulting in a sensor layer of about 12.5 μm (calculated value based on the distance between the knife and support foil and the sensor cocktail composition) after the solvent had evaporated. The crosslinking reaction was catalysed by keeping the foils on a hot plate at 60°C for 1 h before being removed from the glove box. Before the crosslinked sensors could be used they were soaked in a 1 M solution of sodium hydroxide as a deprotection step. The deprotection of the sensors was necessary in order to restore the pH sensitivity to the sensor foil, which was impaired through initial reaction with the crosslinking agent. Once the deprotection is complete, the fluorescence dye returned to its original alkaline form and the fluorescence response is sensitive to small changes.
Figure 46: Sensor cocktail containing pH sensitive dye, Egyptian Blue reference dye and D4 HydroMed polymer dissolved in THF coated onto PET foil support material

Sensor spots were produced from the foils by punching circular holes that correspond exactly to the diameter of the fibre out of the foil, according to the following figure:

Figure 47: Production of a fibre-type pH sensor from sensing foil: 1- The dye in the sensor matrix is applied to a support surface, such as a polymer foil 2+3– The hollow metal cap of the fibre is placed over the sensing area 4+5– The cap is driven through the sensing layer and support foil creating a sensor spot cut-out inside the cap (6) 7+8– The sensing spot is pushed through the fibre cap towards the end where the sensor is trapped by the overhanging edges of the cap, creating a fibre-type sensor
The finished fibres were then attached to the FireStingO2 unit for calibration.

![Image of optical fibres with sensor spots fixed in the fibre-cap](image)

**Figure 48**: Optical fibres with sensor spots fixed in the fibre-cap, produced as shown in Figure 47

### 4.3.1.2. Calibration of the Sensor Spots on Optical Fibres

Before the calibration, the sensors had to be conditioned. In order to do that 100% LED intensity was used to condition the sensors in one-second intervals for 1 h to speed up the inevitable initial bleaching and stabilise the sensor signal for future use. The sensor spots were calibrated by holding the heads of the fibres into beakers filled with buffer of a defined pH that was continuously stirred to ensure mixing. The fibres were submerged until the signal displayed in the FireStingO2 Oxygen Logger software had stabilised before being submerged in the next vessel. Once the calibration was complete the raw data was converted and the $\cot(d\phi)$ was plotted against the actual pH values of the buffers as measured by a pH probe. The resulting calibration curves for all four sensor spots are displayed in Figure 49. The $pK_a$ value of a sensor of this kind varies depending on the matrix it is embedded in. It was determined that the $pK_a$ value is 6.6 for the sensor foil with no added crosslinking agent and 6.3 for 1 and 5% of crosslinking agent in the foils. The 10% foil showed a $pK_a$ of 5.6.
Figure 49: Boltzmann fit of the pH calibration curves to calculate pH from raw values of FireStingO2 readout.

In order to determine the long term stability of the individual sensor dye:crosslinking agent combinations, the sensors were left in an automated buffer circulation tank that continuously circulated through buffers of different pH values. Ideally, a sensor’s calibration curve should remain constant even after multiple cycles. Figure 50 represents the results of the calibration curves obtained during four days of constant pH cycling, for the purpose of the figure one calibration per day was plotted. Each line represents the average of all pH calibration curves obtained on one day, the days are color coded to allow comparison. The sensitivity of each sensor between pH 4 and 10 was determined, showing that each sensor had a sufficient sensitivity to resolve pH changes down to 0.01 pH units or smaller. The response times from pH 4 to pH 10 and vice versa were determined and are documented in Table 11.
Table 11: Sensitivity of each sensor with different crosslinking agent concentrations between pH 4 and pH 10

<table>
<thead>
<tr>
<th>Crosslinking agent concentration (%)</th>
<th>Δdφi (°)</th>
<th>t₀ deprotonation (s)</th>
<th>t₀ protonation (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>48.55</td>
<td>72.9</td>
<td>25.1</td>
</tr>
<tr>
<td>1</td>
<td>45.20</td>
<td>72.9</td>
<td>25.2</td>
</tr>
<tr>
<td>5</td>
<td>45.6</td>
<td>72.9</td>
<td>75.1</td>
</tr>
<tr>
<td>10</td>
<td>46.23</td>
<td>146.5</td>
<td>93.9</td>
</tr>
</tbody>
</table>

With such fast response times, each of the sensors would be suitable for pH measurements in the transketolase reaction, which only has a reaction time of 10 min.

Figure 50: A - Results of stress test for the sensor spots containing 0% crosslinking agent, B - Results of stress test for the sensor spots containing 1% crosslinking agent, C - Results of stress test for the sensor spots containing 5% crosslinking agent, D - Results of stress test for the sensor spots containing 10% crosslinking agent. While the response is consistent, this sensor behaves very differently from the ones with 0, 1 and 5% crosslinking agent. This is due to the sensor being much thicker as the cocktail was already partly catalysing when the foil was produced. The colours of the calibration curves correspond to the days of measurement as follows: Blue – Day 1, Green – Day 2, Orange – Day 3, Red – Day 4. Each point represents an exact pH value for each buffer used for calibration.
The addition of crosslinking into the pH sensors did not improve their stability on PMMA noticeably and was therefore not worth the additional effort of using nitrogen atmosphere to include crosslinking agent and its catalyst in the sensor cocktail. However, it was shown that the use of crosslinking agent is essential when the spots are integrated onto a glass substrate. If no crosslinking agent is used the spots will wash off almost instantly. In order for the spots to adhere to the glass properly, the glass must also be silanised as a pre-treatment. The experiments involving the crosslinking agents were useful for project partners Bogachan Tahirbegi (iX-Factory, Dortmund, Germany) and Susanna Llado (TU Braunschweig), who both worked with glass reactors for oxygen and pH sensors. The crosslinked pH sensor principle that was optimised here was later adjusted for use in Tahirbegi et al. 2016.

4.3.1.3. Initial Side-Entry Reactor Design

The first microreactor design (Figure 51) was manufactured in order to incorporate pH sensors at the Graz University of Technology (TUG) and test the integration of the sensors using a microdispenser. The reactor design consists of two main inlets, two side inlets, an outlet and includes six sensor chambers, each with a diameter of 1 mm. The channel is otherwise 500 µm wide and deep. The reactor layer and lid were laser cut out of 1.5 mm thick PMMA sheets.
Figure 51: Simple multi-inlet reactor for first sensor integration trials: A – channel inlet, B – cut-out for alignment with 3 mm screw, C – cut-out for alignment during bonding process, D – sensor chamber and E microfluidic channel

4.3.1.4. Integration of pH Sensor Spots into a Microfluidic Reactor

The pH sensors were integrated into the reactor as described in the Materials and Methods section. The greatest obstacle to overcome was the clogging of the nozzle by the Egyptian Blue particles in the sensor cocktail. This was counteracted through the integration of a bigger nozzle. It was found that, rather than just spotting the six sensor chambers, the integration of sensors in every length of the reactor channel was more simple and accurate, because the locations of the sensor spot had to be programmed by hand. The microdispenser then found each location, which resulted in clogging of the nozzle due to sedimentation of the Egyptian Blue particles if the sensor spots were located far apart. The continuous spotting made possible by integrating a spot in the centre of every channel length after each turn (see Figure 52), made the nozzle less likely to clog. These are the result of an 8% cocktail, spotted three times.
The sensors’ reproducibility is limited due to the Egyptian Blue particles settling through gravity in the microdispenser nozzle. Therefore, the spots that are produced first have a slightly higher concentration than the ones spotted afterwards. This is shown in the initial phase angle of the sensors. As a result of this, all sensor spots need to be calibrated individually; however, they were all usable.

In order to tune the intensity of the spots, the number of layers spotted onto one spot was increased to six layers. These are the sensors and reactors that were used for the calibration and bonding experiments described in the next section.
4.3.1.5. Bonding of Microfluidic Reactor containing pH Sensor Spots

4.3.1.5.1. Double-Sided Adhesive Tape

The temperature stability of the sensor dyes was unknown when the secondment at TUG was planned, therefore cold-bonding methods for the assembly of the reactor were considered in depth. It was decided to attempt bonding using a double-sided adhesive foil (“Very High Tack Double Sided Tissue Tape” supplied by PSA Solutions, Leicestershire, UK). The foil was laser-cut in the same pattern as the reactor. However, initial trials proved that the serpentine nature of the design were too difficult to align with the laser-cut channels of the reactor. Therefore, additional support struts were introduced to improve handling and alignment of the foil. The struts were cut once one side of the adhesive foil was stuck onto one of the reactor layers, before the second layer was aligned. The foils were cut at high speed (85%) and low power (20%) in order to achieve a clean cut with a minimum amount of scorching to the foil. The pattern the foil was cut in is shown in Figure 53.

![Support strut](image)

Figure 53: Adhesive foil pattern designed to connect the reactor design shown in Figure 51 with a corresponding lid without heat-bonding. The design contains support struts, meaning the channels are not completely pre-laser cut. The foil is first applied to the reactor and then the support struts are cut manually. This preserved the structure of the channel cut-outs during the alignment process.

Once the sensors were integrated and the two reactor layers were adhered to each other, four sensor spots were chosen for calibration, the selected spots are marked Figure 54.
The calibration of the sensors was attempted two days after the bonding was completed to determine the effect of the adhesive foil on the sensors. The calibration showed that the sensors now barely responded to changes in the pH of the buffer flowing through the system anymore. Meanwhile, sensors in a reactor that had been spotted on the same day as these, but had not been foil bonded, were still as responsive as they had been on the day they were spotted. It was suspected, that the solvents in the adhesive tape destroyed the sensors. A calibration attempt with the foil bonded reactor is shown in Figure 55. It was decided that heat-bonding of the reactors should be attempted instead.

Figure 55: pH calibration between pH 5-8 on foil bonded reactor three days after bonding, showing that the sensors are non-functional
4.3.1.5.2. Heat Bonding

The reactor was heat bonded according to Materials and Methods. During this procedure, the pH sensors remained visually unchanged. Four sensors spots were selected, as indicated in Figure 56. The sensors were calibrated from pH 5 to pH 8 and back to pH 5, resulting in the calibration profile shown in Figure 57.

Figure 56: Reactor with integrated pH sensor spots after bonding. The arrows mark the sensor spots that were monitored for the calibration and the TK reaction

Figure 57: Calibrating from pH 5 to pH 8 and back to pH 5 on four channels and four different sensor spots, visual representation of the calibration on reactor (the grey line at 3600 shows a slip in one of the fibres)
The plateau values of this calibration profiles were used to calibrate the sensors, resulting in the calibration curves shown in Figure 58.

Figure 58: Calibrating from pH 5 to pH 8 and back to pH 5 on four channels and four different sensor spots summarised into average values of the plateaus

In order to be able to better compare the results and achieve a reliable calibration curve, the cotan of dphi was calculated and used in order to generate the first calibration curves (Figure 59).
Figure 59: Boltzmann-fit of the calibration data from the pH sensors in the heat bonded reactors shown in Figure 58. All sensors show pH sensitivity and a dynamic range that can be utilised for pH measurements between pH 5.5 and pH 8.

The calibration curves clearly show that the microdispensing of the sensor spots is not homogeneous, or very reproducible at this point in the development. This is due to the Egyptian Blue particles settling in the microdispenser. However, the change in phase shift for each of them is more than sufficient to see small changes in the pH, meaning that the pH sensors are sensitive enough to resolve small pH changes and are therefore suitable for the application in the transketolase reaction. Therefore, while each sensor has to be calibrated, they are very suitable for the application in the transketolase reaction.

A profilometer was used to determine the profile of a sensor chamber before and after the integration of the sensor spot. It showed that the sensors were approximately 500 µm in diameter and 30 µm in height (Figure 60).
Figure 60: Depth profile of a sensor chamber in the microfluidic side-entry reactor without a sensor spot (left) and with an integrated sensor spot (right)
4.3.2. CO₂ Sensors

The carbon dioxide sensors used in this work are composed of a three-layer system. The bottom layer, which is in contact with the polymer, is the reference layer. On top of that is the indicator layer, containing the dye that is sensitive to carbon dioxide and the top layer is a protective layer to protect the sensor. The reference layer of these sensors contains two types of particles: perfluorated Egyptian Blue particles and polystyrol particles coated in di-butoxy-azaBODIPY dye, neither of which are sensitive to carbon dioxide or pH. Due to the choice of PMMA as reactor material and the fact that PMMA stores carbon dioxide internally, it was necessary to find a barrier between the reactor material and the sensor. This is required to accurately detect the carbon dioxide concentration in the solution, whilst minimising the impact of the residual carbon dioxide within the reactor material. To achieve this, the reference particles were dispersed in PEN (polyethylene naphthalate), which is impermeable to carbon dioxide and acts as a barrier between the reactor material and the indicator layer. With a commercially available oxygen meter (FirestingO2) it is possible to measure the phase angle between the wavelengths of the two dyes and the reference particles. The carbon dioxide-sensitive dye in the indicator layer affects the ratio of light emitted by the reference particles through absorbing the emission light differently in presence of carbon dioxide. The protonated form of the indicator absorbs the light emitted by the reference dye while the deprotonated form (no carbon dioxide) takes up the light emitted by Egyptian Blue. This method is referred to as the Inner-Filter Effect, because the carbon dioxide sensitive dye filters the reference light according to the carbon dioxide concentration (see Figure 61). The thickness of this layer, plus the thickness of the protective layer, is what determines the response time of the sensor. The indicator dye layer principle, for making the dye susceptible to changes in carbon dioxide rather than pH, is that of a Mills’ type sensor (Mills et al., 1992). The protective layer is there to protect the indicator dye from H₂S and other acidic components found in air, due to its limited permeability.
However, it is also not easily permeable for carbon dioxide, which greatly increases the response time.

![Figure 61: Visualisation of the Inner-Filter Effect (Schutting et al., 2013)](image)

The development and integration of the carbon dioxide sensors underwent multiple iterations during which the sensor layer format and spot integration evolved multiple times. For this reason, the individual iterations are referred to as a ‘Generation’ of the sensor development in this chapter.

**4.3.2.1. Generation One**

For the first generation, the reference layer was knife-coated with a 1 mm or 3 mm knife onto a PET Mylar foil. This resulted in layers of 0.12 mm and 0.36 mm calculated thickness. After the coating, multiple pressures, patterns and volumes of spraying were tested with different nozzles to test the best way of spotting the indicator dye layers onto the reference layer. Details about the parameters and challenges of this work can be found in Sulzer, 2016 (Sulzer, 2016). During this time, it was crucial to find a method to spray the sensing film in a homogeneous way and to create a layer that allowed enough brightness from the reference layer to come through in order for the signal to be detectable. The minimal requirement for phase shift
between 0 and 100% of carbon dioxide is a difference of 8 dphi (°). The minimal intensity necessary to achieve a reliable result is 10 mV. Figure 62 shows some of the different spots generated in the first attempts.

Figure 62: Carbon dioxide sensors of Generation One before integration in reactor. The carbon dioxide-sensitive layer (purple) is microdispensed onto a knife-coated reference layer (green). The different spot sizes are due to spray patterns and application of multiple layers being tested at this stage of development

Once the indicator dye cocktail was spotted onto the reference layer, a protective layer consisting of 5% Hyflon was knife-coated over the sensor spots to prevent any poisoning of the sensor spots until they could be tested. The schematic for the finished sensor foil is depicted in Figure 63 below:

Figure 63: Sensor foil schematic for carbon dioxide sensors of generation one and two. The reference layer (green) was knife-coated on a support foil, and then the carbon-dioxide sensitive layer was microdispensed onto the reference layer in the form of sensor spots. The sensors were then knife-coated with a protective Hyflon layer to protect them from permanent protonation due to poisoning

In order to be able to test the sensors under defined gas conditions they had to be implemented into a reactor that allows a flow of air (Figure 64). The most promising sensor spots were cut out and glued into the microreactor with large chambers using silicone glue. While the sensors were responsive, the maximal phase angle difference between 0 and 100% CO₂ was only 3
This sensor had a $t_{90}$ response time of 60 s. The minimum for a usable sensor is 8 dphi. If this threshold is not met, the small changes in analyte concentration cannot be sufficiently detected. The phase angle shifts of all tested sensors in this generation can be found in the appendix.

Figure 64: Carbon dioxide sensors of Generation One glued onto a glass slide which was in turn foil-bonded into a commercially available flow chamber reactor (Microfluidic ChipShop) to achieve a controlled environment suitable to maintaining a defined carbon dioxide concentration

### 4.3.2.2. Generation Two

The key to improving this resolution is a thicker indicator layer, which was attempted in generation two. For this purpose, more layers of indicator dye cocktail were sprayed onto the knife-coated reference dye layers and afterwards knife-coated over with a protective Hyflon layer. The indicator cocktail used was 3% thicker than the one used for Generation One, this leads to less splashing during the spotting process and slightly thicker layers that hold 3% more indicator dye molecules, improving the sensitivity.

The most promising spots were glued onto a glass slide and, via adhesive foil, bonded to a Microfluidic ChipShop flow-through chamber lid (Figure 65).
One of the spots that showed the highest difference in phase angle difference of 11.3 dphi (°) between 0 and 100% carbon dioxide was chosen for a calibration. This sensor had $t_{90}$ response time of 100 s. In order to calibrate the spot, a gas mixer was used to generate carbon dioxide concentrations between 0 and 5% (Figure 66). The phase angle shifts of all tested sensors in this generation can be found in the appendix.
Figure 66: Calibration of the most sensitive carbon dioxide sensor spot of Generation Two calibrated in a Microfluidic ChipShop reactor in gas phase containing 0% to 5% carbon dioxide.

The spot shows a large shift in phase angle between 0 and 1% but a low shift between 1 and 5%, meaning its dynamic range is somewhat limited. However, this is the most important range for the application the sensor is intended for. While the calibration shows that the sensors are usable, the resolution of the phase shift with increasing carbon dioxide concentration is still lower than is desirable. This is due to a limited amount of indicator dye molecules in the indicator layer, since only a limited number of indicator layers could be applied to the thin reference layer. If more indicators layers were applied, the signal of the readout would be lower than the detection limit. A thicker reference layer with a larger concentration of Egyptian Blue particles is necessary in order to boost the signal of the readout. Spotting the reference layer as well as the indicator layer would enable this improvement.

### 4.3.2.3. Generation Three

In order to be able to flexibly integrate the carbon dioxide sensors into microfluidic reactors it is necessary to be able to spot all layers individually. Otherwise the option for integration is reduced to sensor chambers or channels wide enough to fix a sensor spot in with silicone glue. This would lead to dead zones and places for bubbles to stick to in solution and compromise
the sensor readout. For this reason, we aimed to spot all of the layers on top of each other, the challenge being the homogeneity of each layer (Figure 67). The sensors were spotted on PMMA slides provided by Microfluidic ChipShop using the microdispenser as described.

![Figure 67: Visual representation of the carbon dioxide sensor spots of Generation Three. The reference layer was microdispensed in a sensor spot pattern onto a PMMA slide. The carbon dioxide-sensitive layer was microdispensed on top of the reference spots at a reduced spot size. Finally, the protective Hyflon layer was microdispensed over the combined sensor spots.](image)

The main difficulty is the dispensing of the reference layer. The particles in this cocktail clog the nozzle of the microdispenser periodically as they gravity-settle. High pressures and frequent cleaning of the nozzle are necessary to ensure smooth, homogenous layers.

After the reference layers were all produced under the same conditions to ensure comparability, the indicator layers were spotted on top of the reference layers with an increasing number of layers to gradually increase the number of indicator dye on top of the reference layer. The more indicator dye molecules are in a sensor layer, the more sensitive the sensor is to small changes in the CO\textsubscript{2} concentration, as there are more molecules that can react to the change. Due to this, the signal intensity is lowered when more indicator dye is present, since the dye filters the emission light from the reference layer. Minimal signal intensity is required in order to detect the phase angle accurately.

In order to determine the ideal balance between signal intensity and sensitivity an array of different amounts of indicator dye layers was spotted onto the reference layers. They ranged from 1 to 30 layers of indicator dye, as shown in Figure 68.
Figure 68: Different amounts of indicator dye layers on the carbon dioxide sensors of generation three. The more layers that are spotted onto the reference layer, the darker the indicator layer appears to be and the less light from the reference layer is emitted back to the fibre, as the rest is filtered out by the indicator dye molecules in the layer above.

These sensors showed a vast improvement in dynamic range between 0 and 100% carbon dioxide in comparison to the second generation of sensors. However, while the signal intensity was sufficient for measurement it was not high enough to be considered useful for practical application. The readout had to happen at an LED intensity of 60%, which might lead to photo-bleaching of the dye if used over an extended period of time. Still, the signal intensity was a vast improvement compared to the one in generation two and phase angle differences between 0 and 100% carbon dioxide of up to 20 were achieved.
Figure 69: Correlation between sensitivity and intensity in the sensor spots of Generation Three showing that thicker indicator layers decrease the signal intensity.

Figure 69 shows the trade-off between signal intensity and sensitivity in proportion to the increasing number of indicator layers. Fifteen indicator layers seem to present a reasonable compromise between the two. Response times for this sensor generation ranged from 5 to 40 min. The phase angle shifts of all tested sensors in this generation can be found in the appendix.

4.3.2.4. Generation Four

In order to increase the brightness and thereby detection limit, the amount of particles that were used for the next attempt at a carbon dioxide sensor was doubled. In addition to that, double the indicator dye was used in the pH sensitive layer. The sensors’ dimensions were 2x2 mm (Figure 70).
Figure 70: Reference layers for homogeneity studies of Generation Four, showing the difference between single-, double- and triple-dispensing of the reference layer.

In order to produce reliable and comparable spots the layer thickness of the reference layer spots had to be as homogeneous as possible. In order to test the homogeneity, the spots produced by microdispensing one, two and three layers of reference material were analysed with a needle-type profilometer (DektakXT, Bruker, Vienna, Austria) that traced the height of each spot in relation to the surface of the substrate they were spotted onto. The results, shown in Figure 71, depict a very homogeneous height distribution for each round of spraying represented by the number of layers.

Figure 71: Profilometer data corresponding with the reference layers shown in Figure 70 of reference layers that were spotted one, two and three times. This shows the homogeneity of the reference layer spots. The spots vertical variations signalise when the fibre was moved from one reference layer to the next.
Figure 72: Complete carbon dioxide sensor spots of generation four on PMMA slide before bonding. These are some of the indicator layers that were spotted onto double-sprayed reference layers

Though this generation of sensors (Figure 72 and Figure 73) looked visually promising due to its homogeneous reference layers, it was not possible to record a calibration curve since the sensors were permanently protonated even after proper storage under carbon dioxide, which was not observed in the previous generations. The poisoning of the sensors is possibly due to chloroform in the cleaning step between the indicator layer and the protective Hyflon layer. If the chloroform had not completely evaporated between the two steps, it could have easily led to a poisoning of the sensors. Another potential source of poisoning is the adhesive tape used for bonding the PMMA slides to the reactor lid, or impurities in the compressed air that is used during spraying. A different explanation is the possible aggregation of the dye in the indicator layer that blocks the acid-base dynamic of the sensor, since double the indicator dye was used in generations four and five.
4.3.2.5. Generation Five

For this sensor generation it was decided that in order to get a more homogeneous signal from the reference layer, the whole reference layer should be coated with the carbon dioxide sensitive indicator layer (Figure 74 and Figure 75). This was in case of a misalignment of the fibre, since in the previous generation, if the fibre was not precisely located above only the carbon dioxide sensitive layer, the signal from the reference layer would be detected more since it was brighter and could compromise the read-out.

Figure 74: Sensor principle of carbon dioxide sensor spots of generation five. The reference layer was completely covered with the carbon dioxide-sensitive layer for more consistent robust readout, as opposed to previous sensor generations.
Figure 75: Carbon dioxide sensor spots of generation five showing increasing thickness of indicator layers over homogeneous and equal reference layer

In this line of investigation, the base concentration inside of the sensor was increased to match the indicator dye concentration of generation 4, as this was another suspected possible reason that the sensors from generation 4 were unresponsive.

The phase angle difference between 0% and 100% carbon dioxide was 25 to 30 dphi, which is more than sufficient in terms of sensitivity for any application. The phase angle shifts of all tested sensors in this generation can be found in the appendix.

However, the stability of the sensors declined quickly and while the signal intensity is good enough to make the sensors usable at 60% LED intensity, the reference layers need improvement in order to boost the signal. The sensor layers of generation four would have made for a better signal intensity, the reference layers of this generation were not as smooth as the ones of generation 4 and it was not possible to add more layers due to the thickness of the cocktail.
Figure 76: Correlation between sensitivity and intensity in the sensor spots of Generation 5 showing that thicker indicator layers decrease the signal intensity.

Figure 76 shows the trade-off between signal intensity and sensitivity in proportion to the increasing number of indicator layers. The integration of four indicator layers seemed to present the best compromise between the two. Any future development of these sensors should use a similar composition as the one presented in generation five. Response times for this sensor generation ranged from 10 to 30 min.

4.4. Conclusion and Suggestions for Future Work

The integration of pH sensor into a serpentine multi-inlet reactor has yielded promising results. The sensors are heat resistant at 110°C for 60 min and show a very good resolution and suitability for the use in the transketolase reaction. The final reactor design will be discussed in the next chapter. In some of the reactors used in the following chapter, only the pH dye tested in this chapter was spotted. In others, a different dye in the same sensor cocktail was used for spotting as well, in order to create a sensor array that covers a wide pH range. This dye has a dynamic range below the other dye and a different pKa value and allows the monitoring of a pH drop below pH 5. This dye was not available at the time of the TUG visit and one of the
chemicals used to synthesise it was unavailable, that is why there are no characterisation experiments using this dye covered in this report. Overall, it can be said that the developed pH sensors are suitable for the monitoring of enzymatic reactions both in their response time and in sensitivity.

These sensors were later used in a microbubble column bioreactor alongside with oxygen and DO sensors intended for monitoring fermentation processes (Maldonado et al., 2018).

The development of carbon dioxide sensors was a challenging task. The most difficult process step is the use of Egyptian Blue particles in the reference layer since the particles clog the nozzle of the microdispenser which leads to inconsistent reference layers, except for the case of generation 4 in which the particles were settled for a longer time. The layers produced like this showed great homogeneity and with the right number of layers also gave a lot of signal in the reference layer. This is crucial in order to measure accurately, since the indicator layer works on the inner filter effect principle and absorbs a lot of the reference layer’s emission.

Overall, the sensors work in principle, though they are too slow (10 to 30 min in gaseous atmosphere, depending on layer thickness) to be used for the transketolase reaction, since the reaction is quite fast in comparison to the sensors in liquid. However, the sensors could be very useful in gas phase. They are fast to respond in wet gases and therefore suitable for monitoring carbon dioxide in the off-gas stream of a fermentation process. It is important to note, that due to the carbon dioxide equilibrium in water, most carbon dioxide that is dissolved equilibrates into hydrogen carbonate at first. The sensors do not detect hydrogen carbonate, which means that in order to detect dissolved carbon dioxide the concentration will have to be quite high. Carbon dioxide sensors may be useful in detecting a carbon dioxide build-up in the reactors, if the system is operated continuously over long periods of time, rather than for the monitoring of a fast reaction. Therefore, the pH sensors were selected for future experiments with enzymatic reactions, both due to their response time as well as their versatility of use for reactions that do not produce carbon dioxide, but show a pH shift. In the future, the sensor composition of the
carbon dioxide sensors needs to be optimised and the production technique needs to be checked for possible introduction of acidic components to the sensor during the spraying of one of the layers, since they are currently not stable in the long term.

Using the insights gained during the development of these two sensor types, a guideline for the development of optical sensors for a microfluidic device was devised. Relevant considerations that need to be made in order to create a successful system were summarised in Figure 77.

Figure 77: Development guidelines for optical sensor integration in a microfluidic device (Gruber et al., 2017c). This diagram shows the logical order of iterative considerations and decisions that need to be made in order to develop a sensor that is suitable for the application it is intended for.

It illustrates the development process towards a robust sensor suitable for its application; starting from choices in reactor material, sensing dye and sensor matrix to considerations regarding integration and assembly of the system (such as bonding and detection methods). The luminescent dye choice depends on the expected analyte range and affects the choice of sensor material. Different sensing schemes and sensor formats are available for different analytes and reactor geometries. The choice of dyes, reactor and sensor materials in turn affects the bonding and detection methods. The best approach is to carefully consider the reaction that will be monitored and plan according to available equipment and materials, as well as expected reaction time and analyte range.
5. pH Monitoring and Adjustment of Enzymatic Reactions Using a Microfluidic Side-Entry Reactor

5.1. Introduction

The pH in a reaction vessel has a great impact on the stability and activity of enzymes, on the ionic state and stability of the substrates, the products and the cofactors in the reaction mixture. These parameters are crucial to enzymatic reactions. For an enzymatic reaction, during which the release or uptake of protons occurs, the pH is particularly important and should be monitored to ensure efficient control of the process and to minimise changes in the reaction over time as pH shifts increase due to continued reaction, since this can affect the yield and quality of the product. In order to accomplish that, robust pH-sensing methods are necessary. A sensor being robust, fast to respond and having the possibility of real-time read out is highly desirable in order to be able to follow the reaction progress. At bench- and industrial-scale, pH is usually monitored and controlled using a commercially available pH electrode with a feedback control system that supplies acid or base to the reaction in a pH stat mode (Jacobsen \textit{et al.}, 2006). As discussed in Chapter 1, the downscaling of this technology is not simple. As the reactor volume is reduced to sub-millilitre volumes, the integration of microelectrodes and needle-type electrodes becomes non-practical or impossible. This is especially the case in microfluidic reaction channels that are narrow and enclosed. This often leaves only at- and offline monitoring through HPLC and GC to ensure the reaction progress. However, in reactions where changes in pH occur, online monitoring is preferable as it shows real-time reaction progress and enables rapid confirmation of reaction and insight into current reaction conditions.

In microfluidic applications, optical sensors are the more frequently used technology in comparison to electrochemical options (Kirk and Szita, 2013). Jezierski \textit{et al.} (Jezierski \textit{et al.}, 2013) applied optical pH sensors in order to observe a pH gradient during free-flow
electrophoresis. They were also used to detect the pH for cell cultivation (Funfak et al., 2009) and to control the pH during microbial fermentations in a batch reactor (Lee et al., 2011). The applicability for real-time monitoring of enzymatic reactions was not shown until 2016 when Ehgartner et al. used pH-sensitive nanoparticles for reaction monitoring (Ehgartner et al., 2016a). In 2017, the work upon which this thesis chapter is based was published (Gruber et al., 2017d). In it, the real-time monitoring of a pH progression of an enzymatic reaction in a microfluidic reactor was demonstrated. The reaction’s pH profile was monitored online through the use of multiple pH sensor-spots along the channel of a microfluidic side-entry reactor (μSER) (Cervera-Padrell et al., 2012). μSERs were previously used to improve the conversion yield of inhibition-prone enzyme reactions (Lawrence et al., 2013). In this thesis, an array of pH-sensitive dyes was used in order to be able to monitor a wide pH range from pH 3.5 to 9. Individual dyes of previously reported work were only able to resolve 2 to 3 pH units (Mousavi Shaegh et al., 2016, Strobl et al., 2015), which limits their applicability in process monitoring. In order to show the capabilities of that specifically developed μSER, well-defined enzymatic reactions during which pH changes occur were chosen. For this purpose, the penicillin G acylase-catalysed hydrolysis of penicillin G (Figure 78), during which a pH decrease occurs and transketolase-catalysed synthesis of ERY (Figure 79), during which a pH increase occurs were selected. The penicillin G acylase is used for the synthesis of 6-aminopenicillanic acid and β-lactam antibiotics (Maldonado et al., 2018, Grundtvig et al., 2017).

Figure 78: Reaction scheme of the penicillin G acylase (PGA) catalysed formation of 6-amino penicillanic acid (6-APA)
The transketolase reaction is a selective two-carbon chain elongation reaction that allows the synthesis of chiral compounds, as discussed in Chapter 2. When hydroxypyruvate is used as a carbon donor, the reaction is rendered irreversible, which drives the reaction towards the product side (Wohlgemuth, 2009, Börner et al., 2017, Žuža et al., 2007). The formation of carbon dioxide as a side-product during the reaction with this substrate leads to an increase in pH due to the equilibrium of carbon dioxide in water, resulting in the formation of hydrogen carbonate.

Figure 79: Reaction scheme of the TK-catalysed synthesis of ERY. ThDP stands for thiamine pyrophosphate.

The work presented in this chapter is a further step towards the use of pH sensors in microfluidic reactors for the purpose of biocatalytic reaction monitoring and the development of µSER-like systems towards process development tools for biocatalysis. For this purpose, the pH sensors described in Chapter 4 were integrated into a microfluidic side-entry reactor (µSER) in order to monitor the pH and adjust the pH through the use of the side entries, according to the enzymes’ ideal operating pH ranges in order to increase reaction yield. The results presented in this chapter were published in Biotechnology Journal under the title “Real-time pH monitoring of industrially relevant enzymatic reactions in a microfluidic side-entry reactor (µSER) shows potential for pH control” (Gruber et al., 2017d).
5.2. Materials and Methods

Unless specified otherwise, chemicals were purchased from Sigma Aldrich (Gillingham, UK) and used without further modification.

5.2.1. Fabrication of the Microfluidic Side-Entry Reactor (µSER)

All components were designed using Solidworks® (Dassault Systems, Vélizy-Villacoublay, France). The reactor consisted of two rigid 1.5 mm poly(methylmethacrylate) (PMMA) layers (RS Pro, Northants, UK). The channels and cut-outs for the connector bars were made using a CO₂ laser marking head (Epilog Laser, Clevedon, UK) and the layers were thermo-compression-bonded (1 h, 110 °C, 400 cNm). Channel dimensions were determined via profilometer (Bruker ContourGT, Coventry, UK). The fibre holder was laser-cut from a 6 mm thick PMMA. Standard connector fittings (P-221, Upchurch Scientific, obtained through VWR, Pennsylvania, USA) were used to attach polytetrafluoroethylene tubing (PTFE, ID 0.75 mm; VWR International Ltd, UK).

5.2.2. Fabrication of pH Sensors

The pH sensors were integrated as described in Chapter 4. Two different pH dyes were employed; dyes number 1 and 3 as presented by Strobl et al. (Strobl et al., 2015) were used for the range of pH 3.5 to pH 6.0 and pH 5.0 to pH 8.5, respectively (Section 4.2.1).

5.2.3. Measurement Setup for pH Sensors

Two FireStingO2 oxygen meters (4-channel phase-shift fluorimeters, Pyro Science GmbH, Germany) were used in combination with optical fibres (1 m length, Pyro Science GmbH, Germany) which were held in place by the precisely fitted 6 mm PMMA fibre-holder plate with 2.5 mm diameter holes above each sensor spot. The plate slotted precisely onto the connector bars and required no further adhesive measures. The sensor read-out occurred for
10 ms in intervals of 1s with an amplitude of 400 mV and a modulation frequency of 2 kHz. Tris-HCl buffers ranging from pH 3.5 to pH 9.0 were pumped through the µSER at the reaction flow rates. The calibration curves were fitted with Boltzmann curve with OriginPro 9.1 (OriginLab Corporation, Northampton, US). Before each use, pH sensors were conditioned in 50 mM Tris buffer pH 7.0 for 60 min at 10 µL min⁻¹.

5.2.4. Transketolase Production and Activity Determination

Transketolase (WT-TK from *E. coli* BL21gold DE3 producing plasmid pQR791) was produced and characterised as described in Chapter 2, section 2.2.4. One transketolase activity unit (U) was defined as the amount of transketolase that catalyses the conversion of 1 mmol of substrate per minute at pH 7.0 and 20 °C.

5.2.5. Transketolase-Catalysed Reaction in a Microfluidic Side-Entry Reactor (µSER)

All enzyme and substrate solutions were adjusted to pH 7.0 and moved with a syringe pump (KDS210, KD Scientific, Holliston, US). Samples were taken after three mean residence times and quenched using 0.1% (v/v) TFA at a 1:10 ratio. When only the primary entries were used, the transketolase reaction was performed with equimolar solutions of HPA and GA (from 100 to 500 mM each) and an enzymatic activity of 3.2 U mL⁻¹. Substrates and enzyme were supplied at a flow rate ratio of 1:1, at a total flow rate of 10 µL min⁻¹. The transketolase was incubated with the cofactors, 2.4 mM ThDP and 9.8 mM MgCl₂, for 1 h before use. All reactions were performed at 20 °C. For side-entry mode, the reactions were performed using transketolase from primary input 1, 600 mM HPA and 100 mM GA from primary input 2, while the side-entries were supplied from a 500 mM GA solution. The exact flow rates and concentrations are summarised in Table 12.
5.2.6. Penicillin Acylase-Catalysed Reaction in the (µSER)

Penicillin G acylase (PGA) was purchased from Sigma Aldrich, Member of Merck Group (Gillingham, UK). The substrate, penicillin G and enzyme were prepared in 50 mM Tris-HCl pH 7.0 buffer. Three samples per run were taken at the outlet for the colorimetric assay quantification. When pH adjustment was performed, the side-entries were used to add pH 8.0 and pH 7.5 Tris-HCl buffers at 50 mM. When only primary inputs were used, the enzyme was pumped at the same flow rates as the substrate solution. For side-entry mode, a solution of 70 mM penicillin G was added through the side-entries at a flow rate of 0.5 µL min\(^{-1}\), while the enzyme with an activity of 3.3 U mL\(^{-1}\) and an initial substrate concentration of 40 mM were pumped in through the primary inputs at a flow rate of 3.5 µL min\(^{-1}\).

5.2.7. Analytics

5.2.7.1. Transketolase Substrate and Product Quantification

ERY and HPA were quantified via HPLC as described in Chapter 2.

5.2.7.2. Colorimetric Assay for the Detection of 6-APA

The activity determination for PGA and the quantification of 6-APA were performed according to Balasingham et al. (Balasingham et al., 1972). 25 µL of sample were added to 175 µL of derivatising solution. The derivatising solution contained 0.5 mL of a 0.5% (w/v) p-dimethylaminobenzaldehyde solution in methanol, 2 mL of a 20% acetic acid (v/v) solution and 1 mL of 0.05 M NaOH in MilliQ water. The absorbance of the solutions in 96-well plates was measured using a Magellan plate reader (Tecan, Männedorf, Switzerland) at 415 nm.
5.3. Results and Discussion

5.3.1. Design of the Microfluidic Side-entry Reactor (µSER)

Inspired by the microfluidic side-entry reactor presented by Lawrence et al. (Lawrence et al., 2013), a new side entry reactor was designed with the goal of making the integration of pH sensors possible. As opposed to the previously presented device, the µSER presented in this thesis consisted of only two layers rather than three, which made the bonding process simpler and integration-friendly with regards to the pH sensors. The reduction by one layer made the optical path to the pH sensors shorter and allowed the read-out from the top of the reactor. The total volume of the reactor was 550 µL, with channel dimensions of 300 µm in depth and 500 µm in width. The µSER had six side-entries for the addition of substrates or buffers of different pH values along the channel length. Similar to the Lawrence et al. design, the side-entries were spaced evenly along the reaction channel (with reaction volumes of ~90 µL between each of the additional inputs). The reactor was laser-cut out of 1.5 mm poly(methylmethacrylate) (PMMA) sheets. The connector bars were also fabricated from PMMA. A schematic and photograph of the assembled reactor is shown in Figure 80.

Figure 80: A – Schematic representation of the microfluidic side-entry reactor (µSER) (top view). The schematic shows the eight sensor positions where the pH was monitored during calibration and enzymatic reactions. B - Photograph of the assembled reactor with an array of pH sensor spots in the centre of each channel length

Figure 81 shows an exploded view of all the µSER components. Each part of the assembly was
laser-cut. The bottom plate containing the channel was thermally bonded with the lid, the connecter bars were attached to the bonded plates using 3 mm diameter screws and the top plate containing the holes for the fibres was held in place by the connecter bars.

Figure 81: A - Exploded view of the µSER  
B – Detailed view of the channel and two sensor chambers

Figure 82: Assembled µSER with the fibres for the pH sensor read-out attached to the top plate above the eight sensor spots that were chosen for monitoring. The slots corresponded to the sensor positions 1 to 8 shown in the schematic representation in Figure 80
Figure 82 shows the assembled reactor with the fibres held in place. The standard straight-tip (ST) connectors (PyroScience GmbH, Aachen, Germany) at the end of each fibre, shown in Figure 83, fit into the fibre holder plates’ slots exactly and therefore did not move during measurements.

Figure 83: ST Connector in fibre holder. The holes of the fibre holder were laser-cut to create a snug fit between the connector and top plate

5.3.2. Characterisation of the µSER

In order to characterise the µSER, the residence time distribution (RTD) was determined using the setup presented by Raimes et al. (2016) (Raimes et al., 2016). Briefly, a UV absorbance detector (D100 ActiPix, Paraytec Ltd, York, UK) with a band-pass filter (254BP12, Omega Optical Inc., Brattleboro, Vermont, USA) was connected to the outlet of the µSER. The detector was used to record the absorbance step change between water and a tracer solution, consisting of 200 mg L⁻¹ L-tryptophan and 4 mM allura red (Sigma Aldrich, Gillingham, UK) in MilliQ water. The tracer solution was moved through the µSER with a syringe pump (neMESYS, Cetoni GmbH, Korbußen, Germany) and the RTD was determined for four flow rates. The cumulative response curve to the positive step change, the F-curve, F(t), was defined as follows:
In order to determine the residence time distribution the data recorded by the Actipix system was smoothed by performing a moving average on the F-curve. The F-curve was then normalised and a numerical differentiation was performed to determine the E-curve. In order to remove the instrument noise, a Savitzky-Golay filter was applied. The peak baseline was adjusted to zero, based on the median value, in order to be able to integrate the area under the peak accurately. The single peak was numerically integrated by using a trapezoidal function.

Figure 84 shows the results for the four flow rates (7, 10, 25 and 50 μL·min⁻¹). The red dotted lines show the respective mean residence times (118, 83, 33 and 16.5 min); the index of the symbol for the time constant, τ indicates the respective flow rate.

Figure 84: Residence time distribution (RTD) of the microfluidic side-entry reactor (µSER). Steady state conditions were achieved between one and two residence times, τ. The red dotted lines show the mean residence times corresponding to the flow rates.
The determined residence times were then compared with the sensor equilibration curves recorded during a penicillin G acylase reaction. The results are shown in Figure 85 for the raw untreated data of the first four sensor spots, containing the pH-sensitive dye for the range pH 5.0 to pH 8.5. This shows that the signal of the sensors stabilised well before two mean residence times, $2\tau$.

![Figure 85: Raw data of the pH sensor spots 1 to 4 during a PGA reaction. The flow rates for both inputs were the same and resulted in a total flow rate of 10 μL min$^{-1}$ in the reaction channel. The red dotted lines indicate one and two theoretical residence times, respectively](image)

5.3.3. pH Sensor Characterisation

In order to monitor the pH, optical pH sensors were dispensed into the reactor’s channel layer of the µSER (Figure 85) before the device was thermally bonded. Forty-one sensor spots were dispensed throughout the reactor in total (fourteen in the sensor chambers and twenty-seven outside of the sensor chambers) as shown in Figure 86. No significant difference between the sensors dispensed in the sensor chamber and in the channels was detected.
The sensor spots had a diameter of 500 µm and a height of 30 µm, as shown in Chapter 4. This presents a significant reduction in comparison with commercially available sensor spots which are typically available from 3 mm in diameter or larger (Ocean-Optics, 2016, PreSens, 2015).

To record a time-course profile of the pH changes during the reactions, the eight positions indicated in Figure 80 were chosen. Those positions were the first and the last sensor position in the reactor and one sensor position immediately before each one of the six additional side-entries. The sensors were dispensed according to the reaction for which the reactor was intended. For the transketolase-catalysed reaction, the sensors contained only the dye with the range between pH 5.0 and pH 8.5. For the penicillin G acylase-catalysed reaction, the dye with a detection range between pH 5.0 and pH 8.5 was chosen for the first four positions and for the last four positions, the sensor dye with a range between pH 3.5 and pH 6.0 was chosen.

The calibration of the sensors was done with an array of buffers ranging from pH 3 to pH 9.
after thermal bonding of the device at 110 °C for 60 min. The calibration plots for these sensors, compiled in Figure 87, show a sigmoid curve typical for pH sensors and exhibit a high reproducibility across the four positions for each type of sensor dyes.

Figure 87: Calibration of eight pH sensor spots in a µSER containing a dye array. The calibration curves show the difference in sensor dyes. Sensors 1-4 contain dye number 3 and sensors 5-8 contain dye number 1 as published by Strobl et al. (Strobl et al., 2015)

The response time ($t_{90}$) for protonation and deprotonation for the pH sensors with dye number 2 (pH 5 to pH 8.5), was 33 s and 250 s, respectively, and with dye number 1 (pH 3.5 to pH 6) 40 s and 80 s, respectively. Furthermore, the long-term stability of the sensors in the reactor was tested by calibrating the sensors on the day the reactor was bonded and after 60 days of proper storage (dry and dark). Figure 88 shows the two calibration curves.
While re-calibration is necessary before each use, unless the reactor is used within a week of the last calibration. Figure 88 shows the long-term stability of the sensors. While sensitivity is lost in the acidic range of the calibration curve, the sensors are still responsive and sensitive after 60 days of storage in the dark.

5.3.4. Real-Time pH Monitoring of the Transketolase Catalysed Reaction

To test if the predicted pH increase during this reaction can be monitored with the integrated sensors, a set of transketolase-catalysed reactions was performed. For these first experiments, only the primary inputs of the μSER were used. The transketolase-catalysed reactions were performed with equimolar concentrations of GA and HPA using three different substrate concentrations (50 mM, 150 mM and 250 mM, respectively). Before the reaction, all solutions were adjusted to pH 7.0.
As can be seen from Figure 89, the pH sharply decreases at the start of the reaction (Position 1). This drop in pH is larger for the higher initial substrate concentrations. Following this drop, the pH shows an overall increase of 0.4 pH (for the 50 mM reaction), 0.7 pH units (for the 150 mM reaction) and 1 pH unit (for the 250 mM reaction). This apparent linear correlation is coincidental, because the pH change is strongly dependent on the initial pH value and the buffer salinity. However, the fact that the reaction pH increases is a definitive indicator of conversion occurring and can be used as an indicator until the offline substrate and product quantification is completed. While this pH increase was expected and has previously been documented with the use of a colorimetric assay using nitrophenol (Yi et al., 2012), the initial decrease of pH has never before been documented. A likely explanation for this rapid decrease is the hydrolysis of the glycolaldehyde dimer, reported by Bell and Hirst (Bell and Hirst, 1939). A similarly sharp drop in pH can be observed during the preparation of the glycolaldehyde
stock solution for these experiments. The offline HPLC analysis confirmed the production of ERY (Figure 90).

![Graph of product and substrate concentrations](image)

**Figure 90**: Offline analysis results of product, ERY and the residual substrate, HPA, measured with HPLC from samples taken at the output for the three different flow reactions

The mean residence time in the µSER was 55 min. At equal enzyme concentrations, this resulted in full conversion for 50 mM and 150 mM, and 95% conversion for 250 mM initial substrate concentration, respectively.

The next step was to now use the reactor in ‘side-entry mode’, by supplying one of the substrates, GA, through the side-entries, to determine if this would increase the yield of ERY or change the pH profile of the reaction. To perform these reactions, transketolase was introduced to the reactor through primary input 1. An initial substrate concentration of 300 mM HPA and 50 mM GA (final concentrations in the reactor) were supplied through primary input 2 (both primary inputs were supplied with a flow rate of 3.5 µL min\(^{-1}\) each). A total concentration of 250 mM GA was added along the channel through the side entries at two different flow rates, 0.25 µL min\(^{-1}\) and 0.5 µL min\(^{-1}\). All the solutions were prepared in 50 mM Tris-HCl buffer pH 7.0. The reactions were performed at 20 °C with an enzyme activity of 3.2
U mL\(^{-1}\). The exact flow rates and concentrations used for each input are summarised in Table 12. The increasing flow rates along the channel length were taken into consideration.

Table 12: Initial concentrations and flow rates used for the transketolase reaction in side-entry mode

<table>
<thead>
<tr>
<th>Input</th>
<th>Substrate concentration at input (mM)</th>
<th>Concentration after mixing (mM)</th>
<th>Flow rate (µL min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary input 1</td>
<td>Transketolase</td>
<td>Transketolase</td>
<td>3.5</td>
</tr>
<tr>
<td>Primary input 2</td>
<td>100 GA + 600 HPA</td>
<td>50 GA + 300 HPA</td>
<td>3.5</td>
</tr>
<tr>
<td>Side-entry 1</td>
<td>500 GA</td>
<td>20.75 / 41.50 GA</td>
<td>0.25 / 0.5</td>
</tr>
<tr>
<td>Side-entry 2</td>
<td>500 GA</td>
<td>20.03 / 40.06 GA</td>
<td>0.25 / 0.5</td>
</tr>
<tr>
<td>Side-entry 3</td>
<td>500 GA</td>
<td>19.37 / 38.73 GA</td>
<td>0.25 / 0.5</td>
</tr>
<tr>
<td>Side-entry 4</td>
<td>500 GA</td>
<td>18.74 / 37.48 GA</td>
<td>0.25 / 0.5</td>
</tr>
<tr>
<td>Side-entry 5</td>
<td>500 GA</td>
<td>18.16 / 36.31 GA</td>
<td>0.25 / 0.5</td>
</tr>
<tr>
<td>Side-entry 6</td>
<td>500 GA</td>
<td>17.61 / 35.21 GA</td>
<td>0.25 / 0.5</td>
</tr>
</tbody>
</table>

For each of the side-entry flow rates, the pH time-course profiles showed the same initial pH decrease at the first sensor spot in the reactor. Throughout the reaction a total increase of 1 pH unit was observed (Figure 91). A final product concentration of 220 mM (± 6.5 mM) for a side-entry flow rate of 0.5 µL min\(^{-1}\) and 230 mM (± 6.8 mM) ERY for a side-entry flow rate of 0.25 µL min\(^{-1}\) was detected via offline quantification. This is a conversion of approximately 95\%.
The experiments showed the monitoring and real-time readout capabilities of the µSER system, even for a fast reaction, such as the transketolase-catalysed synthesis of ERY. In order to further determine the accuracy of the sensors, samples were taken at the outlet and the pH was determined with a pH electrode offline in order to compare the result with the readout of the last sensor in the reactor. The results show that the sensors are sensitive enough to resolve pH changes smaller than 0.1 pH units (Figure 91).
During reactions at substrate concentrations higher than 150 mM (250 mM in Figure 90 and 300 mM in Figure 90), the formation of bubbles in the reaction channel was observed, which is due to the formation of carbon dioxide as a side product. The bubbles passed through the channel without accumulating in the sensor chambers or adhering to the sensors and thus did not obstruct pH measurement.

The time-course profiles did not vary significantly between primary-input mode, where 250 mM initial substrate concentration was introduced all at once and side-entry mode where 300 mM initial substrate concentration was introduced gradually through several inputs. A pH increase of 1 pH unit was observed for both reactions. Both sets of experiments achieved a conversion of 95%. This further indicates the viability of real-time pH profile monitoring for the prediction of conversion efficiency. Since none of the transketolase experiments led to a pH shift to a value beyond the enzyme’s operating range, it was deemed unlikely that any pH adjustment would lead to changes in conversion, and therefore pH control of the reaction through the side entries was unnecessary. A pH measurement of the transketolase-catalysed reaction of the Diels-Alder – transketolase reaction cascade, as reported in Chapter 3, was not attempted due to the destructive effect of 10% acetonitrile in the reaction mixture on the sensors.
5.3.5. Real-Time pH Monitoring of the Penicillin G Acylase-Catalysed Reaction

For the penicillin G-acylase (PGA)-catalysed reaction of penicillin G, two acidic products, 6-amino benzyl penicillanic acid (6-APA) and phenyl acetic acid (PA), are produced as a result of hydrolysis (Figure 78) which leads to a pH shift. The acidity of the products can overcome the buffer capacity and lead to a pH decrease to a range where the enzyme is no longer active. The optimal pH for free PGA from *E. coli* is between pH 6.0 and pH 8.0 (Žuža et al., 2007).

For the experiments, PGA was diluted with 50 mM Tris-HCl buffer pH 7.0 and pumped through primary input 1, and a 40 mM of penicillin G solution (resulting in a 20 mM initial concentration in the reactor) in the same buffer was fed in through primary input 2 at equal flow rates. Four different flow rates were tested: 5, 10, 20 and 40 µL min⁻¹ at each inlet, respectively, leading to a total system flow rate of 10, 20, 40 and 80 µL min⁻¹, respectively. All solutions were adjusted to pH 7.0 prior to their introduction into the µSER. As can be seen in Figure 93, the lower the flow rate, the more of the pH drop occurs within the first half of the reactor. For all flow rates, the difference in product concentration was not statistically significant (Figure 94).
Following the successful characterisation of the reaction in primary-entry mode, two different side-entry mode experiments were performed. The same PGA solution was pumped through the primary input 1, and 5 mM or 10 mM of penicillin G (resulting in a 2.5 or 5 mM initial concentration in the reactor, respectively) in the same buffer were pumped through primary
input 2. Both primary inputs 1 and 2 were supplied with a flow rate of 3.5 µL min\(^{-1}\). Additional penicillin G at concentrations of 2.5 mM or 5 mM respectively was added with a flow rate of 0.5 µL min\(^{-1}\) through the side entries. The exact substrate concentrations and flow rates used for these experiments are summarised in Table 13. The increasing flow rate along the channel length was taken into consideration for this table.

Table 13: Substrate concentrations and flow rates used for the penicillin G acylase reaction in side-entry mode without pH adjustment

<table>
<thead>
<tr>
<th>Input</th>
<th>Substrate concentration at input (mM)</th>
<th>Concentration after mixing (mM)</th>
<th>Flow rate (µL min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary input 1</td>
<td>Penicillin G Acylase</td>
<td>Penicillin G Acylase</td>
<td>3.5</td>
</tr>
<tr>
<td>Primary input 2</td>
<td>10/5</td>
<td>5/2.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Side-entry 1</td>
<td>35/17.5</td>
<td>5.00 / 2.50</td>
<td>0.5</td>
</tr>
<tr>
<td>Side-entry 2</td>
<td>35/17.5</td>
<td>4.83 / 2.41</td>
<td>0.5</td>
</tr>
<tr>
<td>Side-entry 3</td>
<td>35/17.5</td>
<td>4.67 / 2.33</td>
<td>0.5</td>
</tr>
<tr>
<td>Side-entry 4</td>
<td>35/17.5</td>
<td>4.52 / 2.26</td>
<td>0.5</td>
</tr>
<tr>
<td>Side-entry 5</td>
<td>35/17.5</td>
<td>4.38 / 2.19</td>
<td>0.5</td>
</tr>
<tr>
<td>Side-entry 6</td>
<td>35/17.5</td>
<td>4.24 / 2.12</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The pH profiles of the reactions are shown in Figure 95.

Figure 95: Time-course pH profiles of the penicillin G acylase reaction in side-entry mode. For both substrate concentrations, the pH continuously decreases by 2.5 pH units.
In primary-input mode and with a 20 mM initial substrate concentration, a final product concentration of 6 mM to 6.9 mM was achieved (Figure 93). In the side-entry mode reaction, where a 2.5 mM initial substrate concentration was substituted with six additions supplies where 2.5 mM of penicillin G was added resulting in a total substrate supply of 16.3 mM, a final product concentration of 4.4 ± 0.1 mM was observed. Thus, the lower substrate amount led to a lower product formation, which was expected. However, during the side-entry mode reaction, where an initial substrate concentration of 5 mM was substituted with the addition of six times 5 mM of penicillin G, leading to a total substrate supply of 32.6 mM penicillin G when dilution is taken into account, the final product concentration quantified at 6.7 ± 0.1 mM. This did not exceed the maximum primary-input mode result of 6.9 mM where a total substrate concentration of 20 mM was supplied.

This is most likely due to the pH of the reaction dropping below pH 4.5, which is well outside of the enzyme’s ideal operating range. Any further conversion was therefore either prevented by enzyme deactivation or by the 12.5 mM solubility maximum of 6-APA, which is recorded in the substance’s material safety data sheet for a pH of 7, however at a lower pH the solubility maximum is most likely lowered as well. As a result, the side-entries were then used to adjust the pH during the reaction through intervention with buffers to achieve higher conversion.

5.3.6. pH Adjustment of the Penicillin G Acylase-Catalysed Reaction

In order to study the effect of pH adjustment on the product yield of the penicillin G acylase-catalysed reaction, PGA was used as previously described and added through primary input 1, while a 20 mM substrate concentration was supplied to the reactor through primary input 2. A pH adjustment was attempted for reactions with two different initial flow rates (20 and 40 µL min⁻¹). Without pH adjustment, both reactions resulted in a pH decrease below pH 6.0 within the first half of the µSER. In order to adjust the pH to the enzyme’s operating range, pH 7.5
and 8.0 buffers (50 mM Tris-HCl) were pumped through the side entries at constant flow rates (steady-state addition). Two flow rates for both the primary and side-entries were tested to demonstrate the effect of different residence time. The exact flow rates at each input point are listed in Table 14. The reactions were also performed without pH adjustment in order to be able to compare the differences in the time-course profiles.

Table 14: Flow rates and solutions used for the penicillin G acylase reaction in side-entry mode with pH adjustment

<table>
<thead>
<tr>
<th>Input</th>
<th>Input</th>
<th>Flow rate (µL min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary input 1</td>
<td>Penicillin acylase</td>
<td>40/20</td>
</tr>
<tr>
<td>Primary input 2</td>
<td>40 mM Penicillin G</td>
<td>40/20</td>
</tr>
<tr>
<td>Side-entry 1</td>
<td>MilliQ Water</td>
<td>5/2.5</td>
</tr>
<tr>
<td>Side-entry 2</td>
<td>MilliQ Water</td>
<td>5/2.5</td>
</tr>
<tr>
<td>Side-entry 3</td>
<td>50 mM TrisHCl pH 8</td>
<td>5/2.5</td>
</tr>
<tr>
<td>Side-entry 4</td>
<td>50 mM TrisHCl pH 8</td>
<td>5/2.5</td>
</tr>
<tr>
<td>Side-entry 5</td>
<td>50 mM TrisHCl pH 7.5</td>
<td>5/2.5</td>
</tr>
<tr>
<td>Side-entry 6</td>
<td>50 mM TrisHCl pH 7.5</td>
<td>5/2.5</td>
</tr>
</tbody>
</table>

The reaction profile of the reaction without pH adjustment was used to determine the sensor spot and nearest side-entry where the pH dropped below the enzyme’s ideal operating range, which was sensor spot position 5. Therefore, a 50 mM Tris-HCl buffer with pH 8.0 was pumped in through side entries 3 and 4 and a 50 mM Tris-HCl buffer at pH 7.5 was pumped through side-entries 5 and 6, according to Table 14.
As shown in Figure 96, the pH of the system could be maintained at a pH higher than pH 6.0 with the pH adjustment measures presented in Table 14 in order to promote enzyme stability and product solubility. This resulted in higher yields when the dilution factor of the buffers was taken into account. For the reactions without pH adjustment, a product concentration of 7.2 ± 0.2 mM was achieved and a 7.1 ± 0.5 mM concentration was achieved for the 40 µL min⁻¹ flow rate. Using the pH adjustment measures, a product concentration of 6.7 ± 0.3 mM was shown for 20 µL min⁻¹ and a 5.9 ± 0.3 mM concentration was achieved for 40 µL min⁻¹. When the dilution through the side-entries was taken into account, this corresponded to a total product concentration of 9.3 mM for the pH adjusted 20 µL min⁻¹ reaction and a product concentration of 8.1 mM for the 40 µL min⁻¹ reaction, resulting in a yield increase of 29% and 14%, respectively.
5.4. Conclusion and Suggestions for Future Work

A novel microfluidic side-entry reactor (µSER) with microdispensed optical pH sensors was developed. By using two different dyes in the optical sensors, the reactor could be used to detect pH values between pH 3.5 and pH 8.5, which is a broader range than previously reported. A calibration of the sensors was performed after thermal bonding of the channel layer with the lid and showed high pH reproducibility over the entire detection range. This proved the stability of the sensors when exposed to high temperatures, which are typical for the thermal bonding of microfluidic reactors. This robustness makes the sensors highly suitable for the integration into enclosed microfluidic devices. It was possible to obtain accurate time-course profiles of the pH during different types of enzymatic reactions in real-time by monitoring the pH at eight different sensor spots spread out evenly along the channel of the reactor.

The reactor and sensor suitability were validated by monitoring variations of two industrially relevant enzymatic reactions, a transketolase-catalysed reaction and a penicillin G acylase-catalysed reaction. Thereby, it was possible to demonstrate the real-time pH monitoring and establishment of pH time-course profiles for both reactions. Without manual pH adjustment, the transketolase-catalysed reaction showed an increase in pH of up to 1 pH unit while the penicillin G acylase-catalysed reaction showed a decrease in pH of up to 2.5 pH units. When the pH values of the first and last sensor in the reactor were compared for the TK reaction, the L-ERY production was clearly indicated by the pH shift.

The pH shift could serve as an indication of the level of conversion achieved in the reactor and can thus be used for real-time reaction progress monitoring. When pH adjustment was achieved by using the µSER’s side entries in order to provide buffered solutions to adjust the reaction pH to the enzyme’s ideal operating range for the penicillin G acylase reaction, the result was a steady reaction pH and an increase in product yield. An even higher product concentration could be attained if the substrate concentration was increased and the pH adjustment was more drastic, as a pH above pH 8 strongly increases the product solubility. The goal of this
investigation was to show a proof of concept for the pH monitoring and pH adjustment capabilities of the system. The proof of concept has been shown in this chapter.
6. Concluding Remarks and Suggestions for Future Work

The aim of this work was the development of guidelines for the translation of chemo-enzymatic and enzymatic reactions in microfluidic reactors into a continuous flow cascade and the implementation of optical sensors that could be implemented into the reactors for online monitoring in order to achieve insight into the reaction progress.

For this purpose, two cascading reaction systems were chosen as models, one chemo-enzymatic and one two-enzyme cascade. This was done to showcase the different challenges that arose for both types of systems. Furthermore, the monitoring of two parameters was undertaken, which are relevant to both reaction systems being investigated, namely carbon dioxide and pH. Optical pH and carbon dioxide sensor principles were adapted to a microfluidic scale and integration methods suitable for PMMA-based reactors were developed. This allowed insight into reaction progress through observing the change of the parameter throughout the reactor, via several strategically chosen sensor spots along the channel.

This work yielded four successfully peer-reviewed publications in total and a fifth one still in development as of the time of submission of this thesis.

In this conclusion, the main achievements of the project will be broken down by chapter and recommendations for prospective future work approaches are given in a manner corresponding to each line of investigation. Overall, all the main goals for the project were successfully achieved. In order of appearance in the thesis, the achievements are as follows:

- Guidelines for the implementation and optimisation of a cascading reaction system, both chemo-enzymatic and bi-enzymatic, were developed and published in “Conscious Coupling: The Challenges and Opportunities of Cascading Enzymatic Microreactors” in Biotechnology Journal (Gruber et al., 2017b).

- The parameters and considerations needed for successful sensor integration into a microfluidic reactor were summarised in regard to optical sensors for the application in biotechnological processes. This was published as a review in “Integration and
application of optical chemical sensors in microbioreactors” in Lab on a Chip (Gruber et al., 2017c).

- The transketolase-transaminase reaction cascade’s reaction time was decreased drastically from over 20 h, down to only 2 h. This achievement was published in “Enzymatic Synthesis of Chiral Amino-Alcohols by Coupling Transketolase and Transaminase-Catalyzed Reactions in a Cascading Continuous-Flow Microreactor System” in Biotechnology & Bioengineering (Gruber et al., 2017a). During the optimisation experiments, a previously unreported inhibition of the transaminase reaction through the transketolase cofactor thiamine pyrophosphate was discovered.

- This reaction cascade could be further decreased in reaction time through a mixture of immobilised enzymes and free enzymes, or the use of purified enzymes, whether free or immobilised. For this purpose the existing His6 purification procedures would have to be optimised for the enzymes, as initial tests for TAM purification led to a decrease in activity and were therefore not further pursued in this work.

- A multi-input reactor could be designed specifically for the optimised cascade to make the synthesis more user-friendly.

- TAM mutants with higher affinity for L-ERY as a substrate could be used in this reaction cascade to make it more robust and less sensitive to side-reactions.

Through the iterative process of improvements shown during the optimisation of this reaction system it was possible to devise the following guidelines for future optimisations of enzyme-enzyme reaction cascades in a microfluidic reactor (Figure 97). Enzyme-enzyme reaction cascades require a careful reconciliation between the ideal reaction conditions of the first and second enzyme as mismatched reaction conditions can lead to suboptimal performance of the cascade.
To achieve a successful cascade reaction the first enzymatic reaction should first be optimised, this includes considerations regarding the buffer type and concentration, pH, flow rates, residence times and enzyme activities. If substrate inhibition arises, *in situ* substrate supply might be necessary through additional reactor inlets. The introduction of a self-sustaining closed loop could be used to minimise the waste of cofactors and unconverted substrate (Contente and Paradisi, 2018). A further potential difficulty could be product degradation or inhibition, which could be tackled using *in situ* product removal. Once the first step is optimised the next enzyme and substrates can be introduced to the reaction mixture. Here it is important to determine whether the buffer, pH and flow rates match the first reaction. As shown in the TK-TAm case study, substrates, co-substrates, residual substrates from the first reaction and cofactors from the first reaction can all lead to inhibition of the second enzyme. Multiple iterative optimisation steps of reaction conditions may be necessary to achieve a functioning cascade, as was the case for the TK-TAm reaction cascade.

- A proof of concept for the Diels-Alder reaction coupled with the transketolase reaction, as described in Chapter 3, has been shown. While each of these reactions has previously been performed separately, they have never been coupled in sequence, neither in batch
nor in flow. In order to achieve this cascade, a packed-bed reactor was constructed from a commercially available blank column kit and filled with aluminium chloride immobilised on silica gel, the Diels-Alder reaction substrates were diluted in acetonitrile. The Diels-Alder product stream was diluted with enzyme and co-substrate after the packed bed reactor to achieve a flow system. Studies confirmed that the transketolase exclusively converts the R-enantiomer of the intermediate to DCDHP. The microfluidically-coupled reaction yielded an overall conversion of 35% after 200 min of total reaction time.

- The reaction time could be further reduced by performing the transketolase reaction at a higher temperature, such as 37 to 50 °C. While this is unlikely to achieve a higher yield, the transketolase reaction will occur faster and therefore the Diels-Alder reactor could be used close to its maximum capacity with regards to flow rate.

- The reaction should be performed with various DA reaction substrate concentrations, as well as low TK reaction substrate concentrations, as a product inhibition due to the solubility limit of DCDHP might be the reason the overall conversion was never larger than 3.5 mM DCDHP in solution.

- The overall yield could be improved through the use of a different TK mutant, one that has a higher affinity to accept CCA as a substrate. Possible mutants for the use in this reaction were designed in Paul Dalby’s group in the Department of Biochemical Engineering at UCL.
From the insights gained during the process of achieving this proof of concept for the DA-TK cascade it was possible to put together some guidelines to implement other chemoenzymatic reaction cascades in which the chemical reaction is followed by an enzymatic reaction:

![Diagram](image)

Figure 98: Optimisation diagram with key considerations necessary for a coupled chemical (C) and enzymatic (E) reaction cascade. *1 – increase of volumetric activity, *2 - optimisation of reaction time, pH and temperature, or catalyst *3 - re-engineering of the reactor *4 - adjustment of reaction conditions (temperature, pH and dilution of effluent stream of chemical reaction) (Gruber et al., 2017b)

The key issues shown in Figure 98 can lead to poor conversion. In addition to the challenges discussed in Chapter 2, there are some additional considerations to be made when it comes to matching the reaction conditions of a chemical reaction to those of an enzymatic reaction. The choice of solvent in the chemical step is crucial to the feasibility of the enzymatic reaction; the wrong solvent can lead to denaturation or inhibition of the enzyme. As a rule of thumb, the less solvent is present in the enzymatic reaction step, the less inhibition can be expected and therefore a dilution step between the two reactions might be necessary. If dilution does not resolve the inhibition, a complete redesign of the chemical reaction with a different solvent may become necessary.

Chemical reactions can also yield side products that can lead to enzyme inhibition, this can also be avoided through dilution, or alternatively ISPR could be used, if a resin can be found that selectively absorbs the inhibiting compound.
Considerable progress has been made towards implementing carbon dioxide sensors applied to PMMA microreactors, which has not been previously published. The sensor design reliably shields the sensor layer from carbon dioxide diffusion through the microreactor material. The use of multiple layers of reference particles and indicator dye has resulted in highly sensitive sensor spots, at a range suitable for commercial uses involving trace to atmospheric amounts of carbon dioxide. However, the response of the sensors was too slow to be deemed useful for the transketolase reactions presented in the cascading reactions in this work, which is why the carbon dioxide sensor optimisation was not further pursued. The sensors, once the poisoning during the production process has been eliminated, are well suited for use in gas phase monitoring and for the monitoring of reactions where fast response times are not essential.

- The microdispenser cleaning method has to be optimised so no organic solvent is left anywhere in the microdispenser system after cleaning, to avoid contamination of the sensing layer from either of the surrounding layers.
- A thinner reference layer with higher amounts of Egyptian blue reference particles would make the sensors brighter while simultaneously making them more suitable for the implementation into narrow channels.
- A higher concentration of quaternary ammonium ions in the sensing layer will make the sensors more stable and less susceptible to poisoning, as the excess of base will serve as a buffer against any acidic contaminants that would otherwise permanently protonate the sensors.
- The long-term stability of sensors, based on the sensing principle of the adapted carbon dioxide sensors in this work, always stands in balance with the response time. The improvement to the long-term stability of similar
sensors in a fibre principle has been investigated for the application in oceanic carbon dioxide measurements (Fritzsche et al., 2016).

- In this work, pH sensors have been successfully implemented into microfluidic reactors to monitor the progress of enzymatic reactions. An array of sensor dyes was used to achieve a sensitive pH range from pH 3.5 to 8.5 (with a less sensitive total range of 3 to 9). The sensors are heat-stable and show a high pH resolution, which allows an accurate pH measurement, comparable to that of a commercially available pH probe. For use in the reactions described in this thesis, no further improvements to the pH range are necessary. However, if the pH sensors should ever be used as part of the TK reaction monitoring in the DA-TK system, it will be necessary to either dilute the DA product stream at a ratio larger than 1:10, or to shield the pH sensors from the inevitable effect of prolonged exposure to acetonitrile. This could be achieved through a protective layer, but the response time of the sensors would be prolonged in this case. Most likely, diluting the DA reaction stream more is the preferable option in this scenario.

- The pH sensors were implemented into a microfluidic side-entry reactor in order to be able to monitor the reaction progress in terms of pH changes throughout multiple locations in the reactor and furthermore to be able to control the pH through the side-entries into the reaction channel. This reactor setup was used to monitor a transketolase reaction and a penicillin G acylase reaction, generating profiles for both at various flow rates and concentrations. It was then possible to increase the yield of the penicillin G acylase reaction by adjusting the pH in the system through the side-entries. This shifted the pH profile of the reaction into the ideal operating range of the enzyme and thereby showed the benefit of online monitoring in enzymatic reactions at a microfluidic scale. This work was published in “Real-time pH monitoring of industrially relevant
enzymatic reactions in a microfluidic side-entry reactor (μSER) shows potential for pH control” in Biotechnology Journal (Gruber et al., 2017d).

- The optimisation of the penicillin G acylase reaction was not a part of the goals in this work, but could be achieved through the implementation of automatic pH control in the form of a feedback loop between the optical measurement unit and the microfluidic pumps.

- A higher product yield could be achieved in the PGA reaction if the pH was adjusted with a less diluted, lower volume of NaOH, rather than a Tris-HCl buffered solution and if the pH was adjusted up to pH 8 or 9, as the product solubility in that pH range is higher than at pH 6.

- The use of the microfluidic side-entry reactor could be extended to coupled cascade reactions, either to monitor the first reaction (TK-TAm cascade) or the second reaction (DA-TK cascade, considering the stability issues with the pH sensor discussed earlier), or possibly both.

In summary, the goals of implementing and optimising microfluidic continuous-flow reaction cascades and monitoring pH sensitive reactions in reactors with online monitoring capabilities have been achieved. The use of the microfluidic side-entry reactor for pH adjustment in the penicillin G acylase reaction supports the hypothesis that online monitoring is a vital tool for enabling process control in microfluidic reactors to improve the yields in enzymatic reactions.
7. References


BOLIVAR, J. M., LULEY-GOEDL, C., LEITNER, E., SAWANGWAN, T. & NIDETZKY, B. 2017. Production of glucosyl glycerol by immobilized sucrose phosphorylase:
Options for enzyme fixation on a solid support and application in microscale flow format. *Journal of Biotechnology.*


SMITHELLS, R. 1965. The thalidomide legacy. SAGE Publications.


8. Appendix

8.1. Publications, Posters and Conferences

Publications


Conferences

- 4th International Conference IMPLEMENTATION OF MICROREACTOR TECHNOLOGY IN BIOTECHNOLOGY (IMTB), Bled, Slovenia, 2017 – Oral Presentation

- European Symposium on Biochemical Engineering Sciences (ESBES), Dublin, Ireland, 2016 – Oral Presentation
Figure 1: Enzymatic system for the synthesis of chiral alcohols.
1. Motivation

Using hydroxypropene as a ketol-donor in a transesterification reaction leads to the production of CO₂ in the system. This is of advantage since it shifts the equilibrium of the reaction equilibrium towards the product side. The increase in dissolved CO₂ concentration causes a shift in the pH. The drop in pH affects the enzyme activity. Therefore, it is of paramount importance to monitor the pH in the multi-input microfluidic reactor presented here.

\[
\begin{align*}
\text{CO}_2(aq) & \rightleftharpoons \text{H}_2\text{CO}_3(aq) \\
\text{H}_2\text{CO}_3(aq) + \text{Mg}^2+ & \rightleftharpoons \text{Mg}(	ext{HCO}_3)^2+ \\
\text{H}_2\text{CO}_3(aq) & \rightleftharpoons \text{H}^+ + \text{HCO}_3^-
\end{align*}
\]

2. Multi-Input Microfluidic Device

- 2 main inlets
- 6 additional side inlets for the addition of substrates to increase throughput and yield while avoiding or minimizing the effect of substrate inhibition
- Total volume: 550 μL
- Channel dimensions: 300 μm depth, 100 μm width, 3 mm at chamber
- Fabrication: Laser-cut from 1.5 mm PMMA in ~5 minutes
- Thermo-compression bonding at 120°C for 45 minutes with sensors in channels

3. Sensor Integration and Characterisation

- An array of sensors allows us to monitor a pH range from pH 3.5 to 7.5
- Sensors were fabricated via microfluidics at UCL, University of Technology
- Sensor based on the principle of Dual Lifetime Fluorometry
- Synthesis of dye according to Schmidt et al. 2019 [5] at UCL
- Sensor dimensions: 500 μm x 500 μm x 50 μm
- Response time τ = 4 min full protumination pH 3.5 and 13 min full deprotonation pH 7.5

4. pH Monitoring Setup

- Flowing2 system from Pyro Science GmbH used for readout
- Sensors show an intensity of 0-120 mV at an LED intensity of 60%
- Rapid prototyping: Design to chip in < 3 hours [4]
- This setup allows for pH monitoring and better process control

5. Conclusions

- A pH sensor array covering the pH range from pH 3 to pH 7.5 was successfully integrated into a multi-input reactor chip
- Sensors show good stability up to 150°C and an intensity of 48-120 mV at an LED intensity of 60%
- An elegant fiber holder and connector setup was designed to make the readout as easy and user-friendly as possible
- Calibrations show the sensors are homogeneous and confirm the integration method is reproducible

Acknowledgement

This project (EUROSORB) has received funding from the People Programme (Marie-Curie Actions, MEXT-7) of the European Union’s Seventh Framework Programme for research, technological development and demonstration under grant agreement No. 600104

Figure 100: Europt(r)ode XIII, Graz, Austria, 2016 – Poster

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5 Result of Transketolase-Transaminase Coupling in Cascading System

- Applications of new and improved window of operation for the processes conditions
- Metabolic coupling in three-chip system
- Preliminary results show good conversion of XA and EAT to AP within 5 hours of reaction time
- Mass balance to be checked with calibration with synthesized ART for progress

4 Continuous Cascading Reaction System

7 Conclusion

- Design of novel reactor to accommodate the residence time needed for the transaminase reaction step
- New reactor design to aid in multifunctional cascades continuously for improved conversion
- Integration of (pH) reactors for multi-stage operation
- Application of cascade reactor to achieve high yields for the transaminase reaction
- Complex characterisation of the overall system

9 References


Figure 6: Carbon dioxide equilibria in toxin (Left), simple separation reactor design to accommodate sensor spot at the end of the channel (Middle) Design of a pH sensor spot (Right)
8.2. Reactor Designs

Figure 102: Schematic of reactor used for the transketolase reaction in Chapter 2.

Figure 103: Schematic of the micromixer used for transketolase-transaminase coupled reaction in Chapter 2.
Figure 104: Schematic of microfluidic side entry reactor used in Chapter 5.
8.3. Analytical Results

8.3.1. Calibration curves

Figure 105: Calibration curve of hydroxypyruvate

![Graph showing the calibration curve of hydroxypyruvate with the equation \( y = 0.0025x + 0.0001 \) and \( R^2 = 0.9989 \).]

Figure 106: Calibration curve of ERY

![Graph showing the calibration curve of ERY with the equation \( y = 0.0014x - 8E-05 \) and \( R^2 = 0.999 \).]
Figure 107: Calibration curve of S-α-methylbenzylamine

\[ y = 3.0025x - 0.0036 \quad R^2 = 1 \]

Figure 108: Calibration curve of acetophenone

\[ y = 146.4x + 2.2502 \quad R^2 = 0.9995 \]
Figure 109: Calibration curve of 2-amino-1,3,4-butanetriol

\[ y = 228.99x + 0.1014 \]
\[ R^2 = 0.9977 \]

Figure 110: Calibration curve of 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde

\[ y = 2.766x - 0.1481 \]
\[ R^2 = 1 \]

\[ y = 0.257x - 0.0523 \]
\[ R^2 = 0.9999 \]
Figure 111: Calibration of 1-(3,4-dimethyl-3-cyclohexen-1-yl)-1,3-dihydroxypropan-2-one

8.3.2. Peaks in HPLC and GC Chromatograms

Figure 112: Chromatogram of hydroxypyruvate, peak at residence time 8.4 min

Figure 113: Chromatogram of ERY peak at residence time 11.5 min
Figure 114: Chromatogram of S-α-methylbenzylamine, peak at residence time 3.6 min

Figure 115: Chromatogram of acetophenone, peak at residence time 7.8 min

Figure 116: Chromatogram of hydroxybutyrate 2-amino-1,3,4-butanetriol after TK-TAm coupled reaction, ABT peak at residence time 6.0 min
Figure 117: Chromatogram of 3,4-dimethyl-3-cyclohexene-1-carboxaldehyde, peak at residence time 18.5 min

### 8.4. Boltzmann fit

<table>
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<tr>
<th>Modell</th>
<th>Boltzmann</th>
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<tr>
<td>Gleichung</td>
<td>$y = A2 + (A1-A2)\frac{1}{1 + \exp((x-x0)/\delta x)}$</td>
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<tr>
<td>Kor. R-Quadrate</td>
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Figure 118: Boltzmann fit for calibration curves shown in Figure 49

### 8.5. Carbon Dioxide Sensor Sensitivity and Intensity Data

Table 15: Generation One – data acquired with 100% LED
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<th>(CO_2) (dphi)</th>
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Table 16: Generation Two – data acquired with 100% LED
<table>
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<tr>
<th>Spot</th>
<th>$N_2$ (dphi)</th>
<th>$CO_2$ (dphi)</th>
<th>$\Delta$dphi (°)</th>
<th>Intensity (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3mmb1</td>
<td>25.5</td>
<td>30.9</td>
<td>5.4</td>
<td>&gt;40</td>
</tr>
<tr>
<td>3mmb2</td>
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<td>30.5</td>
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<td>&gt;40</td>
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<tr>
<td>3mmb3</td>
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<td>29.8</td>
<td>4.5</td>
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</tr>
<tr>
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<td>29.1</td>
<td>5.0</td>
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<tr>
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<td>29</td>
<td>4.0</td>
<td>&gt;30</td>
</tr>
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<td>3mmd3</td>
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<td>3.9</td>
<td>&gt;30</td>
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<td>3mmd4</td>
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<td>&gt;30</td>
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<td>3.4</td>
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Table 17: Generation Three – data acquired with 60% LED

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<tr>
<th>Spot</th>
<th>$N_2$ (dphi)</th>
<th>$CO_2$ (dphi)</th>
<th>$\Delta$dphi (°)</th>
<th>Intensity (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>b5x</td>
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<td>8.3</td>
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<tr>
<td>b8x</td>
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<td>27.9</td>
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<tr>
<td>b8x</td>
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<tr>
<td>b10x</td>
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<td>Spot</td>
<td>( N_\text{2} ) (dphi)</td>
<td>( \text{CO}_\text{2} ) (dphi)</td>
<td>( \Delta \text{dphi} ) (°)</td>
<td>Intensity (mV)</td>
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<td>-----------------</td>
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Table 18: Generation Five – data acquired with 60% LED