Peter Panjan

INNOVATIVE MICROBIOREACTORS AND MICROFLUIDIC INTEGRATED BIOSENSORS FOR BIOPHARMACEUTICAL PROCESS CONTROL
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INNOVATIVE MICROBIOREACTORS AND MICROFLUIDIC INTEGRATED BIOSENSORS FOR BIOPHARMACEUTICAL PROCESS CONTROL

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UNIVERSITY OF OULU, OULU 2018
Biopharmaceuticals are growing in medical and economical significance, replacing several chemically synthesized pharmaceuticals and providing new treatments for serious diseases. Research within the biopharmaceutical field is expensive and labor intensive with the majority of work featuring diverse bioprocesses. Miniaturization of bioprocesses into microbioreactors, miniaturized bioprocess vessels for diverse experiments, reduces the amount of necessary reagents as well as time and labor due to the possibility of multiplexing. A crucial step of research is analytics, commonly performed by expensive and time consuming standard laboratory based analytical tools that require larger sample volumes that than microbioreactors can provide. Biosensors are analytical devices that can provide highly sensitive and selective online detection (in situ) and lend themselves to miniaturization. Biosensors detecting glucose, lactate, pyruvate and galactose were developed and investigated herein. 3D printing and laser ablation were used as fabrication techniques, enabling development of unit operations (mixing and pumping) necessary for biosensor integration into microbioreactors. A 3D printed microbioreactor was developed with integrated glucose and optical density sensors for the cultivation of yeast where the biosensor was found to provide critical online data of glucose concentration, thus enabling bioprocess control. Additionally, molecularly imprinted polymers were investigated as novel recognition components due to their increased stability compared to biological molecules within bioprocess environments. A molecular imprinted polymer for folic acid was developed and tested as a sorbent in a microfluidic solid phase extraction system for detection.

**Keywords:** biopharmaceuticals, bioprocess, biosensors, microbioreactors, microfluidics
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Tiivistelmä

Asiasanat: biofarmaseuttinen lääkevalmiste, bioprosessi, biosensori, mikrobioreaktori, mikrofluidiikka
This thesis is made of sweat, blood and lots of hot glue.
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10.10.2017

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**Abbreviations**

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>% (m/m)</td>
<td>mass percentage</td>
</tr>
<tr>
<td>% (m/V)</td>
<td>mass per unit volume percentage</td>
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<tr>
<td>% (V/V)</td>
<td>volumetric percentage</td>
</tr>
<tr>
<td>4VPy</td>
<td>4-vinylpyridine</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>ADC</td>
<td>analog to digital conversion</td>
</tr>
<tr>
<td>AIBN</td>
<td>azobisisobutyronitrile</td>
</tr>
<tr>
<td>B</td>
<td>binding capacity</td>
</tr>
<tr>
<td>$B_{\text{MIP}}$</td>
<td>binding capacity of a molecularly imprinted polymer</td>
</tr>
<tr>
<td>$B_{\text{NIP}}$</td>
<td>binding capacity of a non-imprinted polymer</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>c</td>
<td>concentration</td>
</tr>
<tr>
<td>$c_{\text{B}}$</td>
<td>concentration in buffer</td>
</tr>
<tr>
<td>CE</td>
<td>counter electrode</td>
</tr>
<tr>
<td>CFD</td>
<td>computational fluid dynamics</td>
</tr>
<tr>
<td>$c_{\text{fin}}$</td>
<td>final concentration</td>
</tr>
<tr>
<td>$c_{\text{M}}$</td>
<td>concentration measured in sample</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>COC</td>
<td>cyclic olefin copolymer</td>
</tr>
<tr>
<td>CV</td>
<td>cyclic voltammetry</td>
</tr>
<tr>
<td>D</td>
<td>diffusivity</td>
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<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DVB</td>
<td>divinyl benzene</td>
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<tr>
<td>e.g.</td>
<td>exempli gratia</td>
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<tr>
<td>EGDMA</td>
<td>ethylene glycol dimethacrylate</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>etc.</td>
<td>et cetera</td>
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<tr>
<td>FA</td>
<td>folic acid</td>
</tr>
<tr>
<td>FAD</td>
<td>flavineadeninedinucleotide</td>
</tr>
<tr>
<td>FDM</td>
<td>fused deposition modeling</td>
</tr>
<tr>
<td>FFF</td>
<td>fused filament fabrication</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>GOx</td>
<td>glucose oxidase</td>
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HPLC  high pressure liquid chromatography
IF  imprinting factor
J  diffusive flux
LED  light emitting diode
LOC  lab on a chip
LOD  limit of detection
m  mass
MAA  methacrylic acid
MB  Meldola Blue
MgCl2  magnesium dichloride
MIP  molecularly imprinted polymer
n0  initial molar amount
n1  final molar amount
NADPH  nicotinamide adenine dinucleotide phosphate
NMP  methyl-2-pyrolidinone
OD  optical density
PB  Prussian Blue
PBS  phosphate buffered saline
PC  polycarbonate
PDMS  polydimethylsiloxane
PdTFPP  platinum tetrakis pentafluorophenylporphiphin
PLA  polylactic acid
PMMA  poly(methyl methacrylate)
pO2  partial oxygen pressure
POC  point of care
R1-4  packed bed microbioreactors
RE  reference electrode
RNA  ribonucleic acid
RT  room temperature
RTD  residence time distribution
S  optical signal
SELEX  selective evolution of ligands by exponential enrichment
SLA  stereolithography
SLS  selective laser sintering
Smax  maximum optical signal
Smin  minimum optical signal
SPE  screen printed electrode

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<td>SPE</td>
<td>solid phase extraction</td>
</tr>
<tr>
<td>TPP</td>
<td>thiamine pyrophosphate</td>
</tr>
<tr>
<td>TPTBPF</td>
<td>palladium(II) meso-tetra(4-fluorophenyl) tetrabenzoporophyrin</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet light</td>
</tr>
<tr>
<td>V</td>
<td>volume</td>
</tr>
<tr>
<td>VBTMAC</td>
<td>vinylbenzyltrimethylammonium chloride</td>
</tr>
<tr>
<td>WE</td>
<td>working electrode</td>
</tr>
<tr>
<td>x</td>
<td>distance</td>
</tr>
<tr>
<td>YPD</td>
<td>yeast peptone dextrose cultivation medium</td>
</tr>
<tr>
<td>µTAS</td>
<td>micro total analysis system</td>
</tr>
<tr>
<td>$\Phi_n$</td>
<td>diffusion flow rate</td>
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Original publications

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1 Review of the state of the art

1.1 Introduction

Biopharmaceutical industry is a branch within the pharmaceutical field that deals with the biotechnological products for human health [1], manufactured in, extracted from or semi-synthesized in biological systems [2]. Since the introduction of the first recombinant insulin into the market in 1980s [3], biopharmaceutical drugs changed the prospects of patients suffering from severe diseases including cancers [4] and immune-mediated chronic diseases [5]. More than 200 biopharmaceutical drugs have been approved for marketing so far, corresponding roughly to half of global pharmaceutical expenditures due to extreme development and production costs [6]. Cell cultures are an essential tool in the biopharmaceutical industry. They are used for drug screening and testing within pre-clinical trials thus reducing the amount of animal testing, but also serving as microbial factories for the production of biopharmaceuticals [7]. Cultivation of cells is critical; identifying a production strain and optimal medium and fermentation conditions requires a large amount of work and resources [8].

Miniaturization of analytical research tools offers reduction in turnaround times and costs as a result of reduced hardware and material requirements [9]. Eichler et al. for example report the development of a miniaturized 96-well microtiter plate-like system for drug screening for neuroblastoma and glioblastoma multiforme, which are severe brain cancers [10]. The developed analytical platform featured a small footprint allowing 96 experiments to run in parallel, whilst the small volumes required only small amounts of reagents, cells and invaluable pharmaceutically active compounds [10]. Such advantages account for more economical and quick experiments to be performed for biopharmaceutical applications and other fields. Microfluidics is a technology that enables miniaturization, automation and multiplexing laboratory equipment and experiments of crucial importance for biopharmaceutical research [11]. With the development of technologies for building micro-structured devices in a controlled and repeatable manner emerged a possibility of integrating one or more laboratory functions on a microfluidic chip (Lab on a chip, LOC) [12]. A group of such functions is bioprocessing, and microfluidics which utilized for such purposes can be characterized as microbioreactors [12]. A crucial part of biopharmaceutical developmental research is in detection analytics of which all aspects from sampling
to detection can be integrated into a chip, yielding the concept of a micro total analysis system (µTAS) on which microfluidic clinical point of care (POC) devices are based [13]. Integration of sensors into microfluidic devices allows online, real-time measurements of bioprocess parameters like temperature, pH and dissolved oxygen [14], however biochemical analysis remains in the domain of standard analytical equipment that only works offline and requires relatively larger samples than microbioreactors can provide and is both resource and labor intensive.

1.2 Microfluidics

Microfluidics refers to fluid handling technologies with sub-millimeter features and low, sub-milliliter internal volumes [15]. Originally developed for inkjet printing, microfluidics is currently used in analytical and synthetic chemistry, biotechnology, biopharmacy and other fields [15] as it boasts small footprints of devices and reduces the consumption of reagents and samples [13]. Microfluidic devices often strive towards automatization, multiplexing and high throughput of research [11].

Due to the small sizes of channels and features, microfluidic flow behaves differently to the large scale as surface tension including capillary effects, energy dissipation and fluidic dissipation dominate the system [16]. As the channels and features are of sub-millimeter sizes, laminar regimes govern the flow patterns (typical Reynold’s numbers in microfluidic systems are very low) [16]. In contrast with large scale flows, turbulence rarely occurs within microfluidic channels, making large scale convective mixing actuated by turbulent vortices difficult. Instead, mixing is diffusive and can be enhanced by microfluidic features that enlarge the contact surface between the liquids to be mixed [17].

In order to integrate laboratory functions into a microfluidic chip (LOC principle), pumping, mixing and valving functions have to be reassured [13].

Microfluidic chips are most often made of glass, polydimethylsiloxane (PDMS), poly(methyl methacrylate) (PMMA), polystyrene [8] and cyclic olefin copolymer (COC) [18]. Frequently reported fabrication techniques involve micro-milling [19], laser engraving (L) and molding [18], whilst recent improvements in resolution of 3D printing have enabled its use for microfluidic device fabrication [20]. Materials and methods for microfluidic chip fabrication are chosen based on necessary bio- and chemical compatibility, size, resolution of features as well as optical and thermal bioprocess demands.
1.3 Microbioreactors

Bioreactors are devices with enclosed chambers for biochemical reactions [21] used in the areas of bioprocessing, tissue engineering and regenerative medicine for three distinct applications: production of biologic end-products (production bioreactor), cell or stem cell expansion (cell bioreactor) and tissue engineering (tissue bioreactor) [22]. Miniaturization of bioreactors to micrometer scale would facilitate new applications such as biosensors, micro-bioreactor arrays or microbiological assay kits [21].

Microbioreactors are miniaturized bioreactors of sub-milliliter internal volumes [21] used for research within biopharmaceutical field. They are used for drug screening [23], preclinical studies [24] as well as finding optimal production bioprocess conditions [8]. Microbioreactors have the advantage over large scale bioreactors for great reduction of required reagents and chemicals due to low chamber volumes. Miniaturization also accounts for small footprint and required resources, enabling multiplexing of experiments. Favorable transport mechanisms speed up the reactions, thus allowing high throughput systems [12]. Furthermore, microbioreactors improve the process control due to the low internal volumes, allowing the optimized production of sensitive high value biopharmaceutical products. Microbioreactors are usually produced by the same micro-fabrication techniques as the microfluidic chips (described in Chapter 1.2).

Microbioreactors can serve as platforms for biotransformations with immobilized whole cells [25], enzyme-bearing particles [26] or enzymes immobilized directly onto the walls of microbioreactors [27]. Biotransformations have the advantage over chemical reactions due to their environmental sustainability, selectivity and enantiomeric purity of the products, however they require a large number of experiments to meet the bioprocessing conditions and are thus greatly enabled by microbioreactor technology [25].

Research of clinical embryology involves exposure of embryos to diverse conditions including different pH values and growth hormone concentrations; mimicking these conditions involves copious amounts of labor and reagents, whilst Perrozio et al. report conducting the same experiments in specialized microbioreactors that allowed parallelization and automatization, making a great number of experiments technically feasible [28].

The majority of bioprocess technology for drug or precursor production operates in fed batch [29] or continuous [30] mode that requires integrated actuation systems, mixing and pumping in particular. Other specific requirements
like sterility and process conditions have to be met by the whole system, including the integrated system-compatible components.

### 1.4 Biosensors

A biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is in a direct spatial contact with a transducer element. [31]. A scheme of an ideal biosensor is depicted in Figure 1. It uses specific biochemical reactions mediated by isolated enzymes (referred to as enzymatic biosensors, catalytic biosensors or simply biosensors), biologically derived receptors (affinity biosensors), tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals [32]. The biological recognition component is often a molecule or particle that has specific binding affinity towards a relevant analyte. Enzymes, antibodies, aptamers and artificial receptors can be generally considered for the development of a biosensor. The choice of an appropriate transducer is dependent on the recognition component and application as well as on the available readout technology. Most transducing technologies can be divided into electrochemical [31], optical [33], gravimetric (mass sensitive) [34], magnetic [35] and pyroelectric (temperature sensitive) [36]. Biosensors are designed to rapidly and quantifiably detect the concentrations of target analytes in the media they are exposed to. Though limited to a single analyte or a group of analytes, biosensors offer advantageous selectivity, sensitivity and simplicity of detection over the standard analytical equipment (liquid and gas chromatography etc.) [37]. On the other hand, a biosensor is only as robust as its most fragile component and hence the biological recognition components pose several challenges (I).

The first biosensor was made by Clark and Lyons in 1962 by coupling an oxygen sensitive electrode (transducer), inner oxygen semipermeable membrane and glucose oxidase (biological recognition component) [39]. From 1962 onwards biosensors have been utilized in diverse clinical [40–42], biotechnological [43–45], environmental [46–48] and several other applications, targeting a diverse range of analytes from small molecules [49], DNA [50], proteins [51] or to even whole microorganisms [52].
Fig. 1. General scheme of a biosensor. Analyte is quantifiably detected by a biological recognition component, followed transducer's translation into readable signal by appropriate electronics.

1.4.1 Biological recognition components

Biosensors are often based on natural receptor molecules that feature highly selective affinity towards a targeted analyte. Most recognition components can be divided between the following groups: enzymes (oxidases, dehydrogenases etc.), immuno-agents (antibodies, antibody fragments etc.), nucleic acids (DNA and RNAs) and artificial receptors (plastic antibodies).

One of the most important groups of biological recognition components are enzymes [53], characteristic for their consumption of the targeted molecule and occurrence of products and byproducts (catalytic biosensors). One of the easiest groups of enzymes for signal transduction are oxidases; as the activity of the enzyme and hence the consumption of oxygen is dependent on the analyte concentration, an oxidase can be coupled with an oxygen sensor (electrochemical or optical) to monitor the decrease of pO₂ from which the analyte concentration can be calculated. Glucose (II), lactate [55] and alcohol [56] are among the targets that can be detected using oxidase based biosensors.
Dehydrogenases in contrast to simplistic oxidases often require several cofactors like NADPH and FAD to function optimally but despite represent an important group of enzymes for biosensing; alcohol [57], glycerol [58], lactate [59], pyruvate [43] and several other biosensors have recently been reported as based on dehydrogenases.

The utilization of other enzyme groups for biosensor applications is occasional. For example: a transferase has reportedly been used for captan detection [60], a hydrolase for organophosphate nerve agents detection [61], a lyase for capsaicin detection [62] and ligases as an aiding component for DNA detection [44,63].

Antibodies are proteins produced by natural immune systems with specific recognition capabilities of diverse molecules (antigens). Natural and engineered antibodies are used in the enzyme-linked immunosorbent assays (ELISAs) for detection of a variety of molecules at very low concentrations. In recent years, translation of the biological event of antigen-antibody interaction into a quantifiable signal has been explored yielding several transducer technologies that enable antibody-based (affinity) biosensors (optical [64], magnetic [35] and mass-sensitive detection). Hence, antibody based biosensors are only limited by the selection of natural or transgenic antibodies; each available antibody can serve as a basis for the development of a biosensor. Diverse affinity biosensors have been reported in various stages of development in the fields of therapeutic drug monitoring [65], environmental monitoring [66], metabolic engineering and several other fields [67].

The selection of biological recognition components for biosensors is not limited to the naturally existent molecules; engineered oligopeptides as well as oligonucleotides (for DNA and RNA detection, made by selective evolution of ligands by exponential enrichment, SELEX [68]) – aptamers – can target novel analytes. As a biosensor inherits the stability of the fragile biological recognition component, aptamers are also appreciated for their improved thermal and chemical stability [69].

Further extending the stability and target range of biosensors are synthetic polymeric receptors or molecularly imprinted polymers (MIPs). MIPs are polymeric materials with specific bonding sites obtained by self-copolymerization functional monomers and crosslinkers around a template molecule, yielding cavities of appropriate shape and exposed complimentary functional groups to adsorb the target [70,71].

MIPs are commonly used as a sorbent for solid phase extraction (SPE), aiding the detection that follows by highly selective pre-concentration of the analyte from
the sample, thus increasing the sensitivity of the standard measurement methodology (e.g. HPLC) [72].

As the optical properties of certain forms of MIPs change when binding of the target molecule occurs, MIPs can be utilized as highly stable and specific recognition components for optical biosensors [73]. By adding magnetic nanoparticles into the polymerization mixture during the MIP synthesis, the magnetic detection assays for biopharmaceutical, clinical, environmental and food applications [74] can be developed. Jolly et al., for example, report the development of a novel MIP-aptamer hybrid against the prostate specific antigen; coupled with electrochemical impedance spectroscopy, the hybrid reportedly features three-fold lower limit of detection in comparison with a bare aptamer [75].

1.4.2 Transducers

A transducer is a material and technology that translates the biological binding event into a quantifiable signal [76]. A choice of a transducer is linked to the chosen or engineered recognition component and application. Within the scope of this research, optical and electrochemical transducers were used.

Optical detection in analytical chemistry is common; light absorbance, luminescence or refractive index detection are regularly used in spectrophotometers, HPLC detectors, microtiter plate readers etc. Optical chemical sensors are well established technologies for monitoring of pH, partial oxygen pressure, temperature and several other variables and are successfully being integrated into microbioreactors [14]. Such sensors are the basis for optical biosensing technology, for instance, a combination of an oxygen sensitive dye and an oxidase can form a biosensor (II).

Biosensors based on electrochemical transducers measure the analyte concentrations by monitoring the changes of electrical properties [76]. An electrochemical biosensor usually consists of working, reference and counter electrode in a three electrode system whilst in a two electrode system the reference and counter electrodes are combined into a single one. The types of electrochemical detection involve voltammetry and amperometry, impedancetry, conductometry and potentiometry [76].

Amperometry is an electrochemical technique where the currents generated by a biosensor are monitored, providing real time data on the analyte concentration and are commonly used for catalytic biosensors [76]. Voltammetry techniques involve monitoring the current response when a range of potentials is being applied
Impedance detection measures the resistive and capacitive properties of materials when perturbed by a small amplitude sinusoidal potential [76,77]. Impedance detection is primarily used for affinity biosensors as it is capable of detecting electron transfer at high and mass transfer at low frequency (e.g. binding of an antigen to immobilized antibody) [78].

Conductometry monitors the changes in electrical conductivity of the sample solution and is commonly used in combination with enzymes causing ionic strength changes (a change in conductivity as a result of an enzymatic function) [76].

Potentiometry is a passive methodology of measuring a potential of an electrochemical cell (e.g. across a membrane of a pH meter, dividing the sample and reference solutions) with drawing only negligible currents [76].

Regardless of the electrochemical detection method, a biological recognition component has to be immobilized onto the electrode surface. The most common techniques are entrapment into a gel or a polymer, surface adsorption, covalent binding and electrostatic immobilization [76].

Electrochemical biosensors can be grouped into three generations. The first generation biosensors are based on oxygen sensitive electrodes, the second generation are mediator based whilst the third generation are enzyme electrode-coupled biosensors [76]. All electrochemical biosensors featured within this thesis are second generation, using Prussian blue [49] or Meldola blue dyes as mediators [43].

1.5 Analytics in microbioreactor bioprocessing

Microbioreactors boast numerous advantages in parallelization, high throughput screening, enhanced mass and heat transport, decreasing the consumption of reagents, but they can offer only low amounts of products due to operation in the micro-scale. Microbioreactors require monitoring of the same essential factors as in large scale bioprocesses – temperature, pH, cell mass, dissolved oxygen and carbon dioxide [79]; sensors for online monitoring of listed variables have been developed and are described in Chapter 1.5.1.

In order to perform chemical and biochemical analysis of bioprocesses in microbioreactors, standard analytical (full size) equipment, e.g. HPLCs and GCs, remains largely unrivaled [25,30]. Such analytics is both labor and equipment
intensive and can provide steady state analysis at best. Furthermore, microbioreactors offer only a small amount of invaluable samples; Perozzielo et al. reported that dilutions of samples were necessary to measure the pH within the microbioreactor chip, compromising not only the number of measurement points but the sensitivity as well [28].

1.5.1 Online analytics using integrated sensors

Integration of physical sensors for the bioprocess environment monitoring including temperature, pH and partial pressure of dissolved oxygen (pO₂) is common [14]. Hence, the major challenge of integration is miniaturization of sensors and ensuring that the readouts are representative and meaningful [14].

There are several options for temperature measurements and all techniques involve monitoring the change of a certain material property with temperature. Thermocouples are the simplest temperature sensors that involve detection of charge allocation which changes with temperature. The accuracy of such sensors is up to 1 K [80]. The most accurate temperature measurement system is a thermistor where the change of resistivity of the material is dependent on the temperature it is exposed to. Such sensors do not need frequent calibrations and can easily detect the changes in range of 1 mK [81]. Integration of both thermocouples and thermistors into the microbioreactors has to involve direct or indirect contact with the process fluid, essentially requiring only a mechanical integration into the spot where the temperature has to be monitored.

Detection of pH can be done optically or electrochemically. For example, Pereira Timbo et al. reported a thin layer luminescence pH sensor based on bromocresol purple dye applied in a sol-gel onto a silica substrate [82]. Yoon et al. on the other hand report an electrochemical pH two electrode system (polyaniline nanopilar array electrode and Ag/AgCl electrode) [83] whilst Aigner et al. report the application of rhodamines as indicator dyes in fluorescent pH sensors [84]. Both solutions could potentially be integrated into a microbioreactor based on described specifications.

A typical electrochemical dissolved oxygen sensor is a Clark electrode that uses a platinum catalytic surface to monitor the electron transfer (current) due to reaction of oxygen and hydrogen into water [85]. Detection of dissolved oxygen can also be done by using dyes, like PdTFPP (II) or platinum(II) meso-tetra(4-fluorophenyl) tetrabenzoporphyrin (PtTPTBPF) [86]. Due to the stability and possibility of miniaturization, Tahirbegi et al. report a successful integration of
optical pH and oxygen sensors into microfluidic analytical system for a quick
detection of pesticides [86].

1.5.2 Standards of practice in small molecules monitoring

Bioprocess control is dependent on analytical data consisting not only on physical
parameters like temperature and optical density, but also of small molecules,
including essential substrates and products. Typically, the concentration of
biopharmaceutical products or intermediates and nutrients, especially glucose, is
monitored using the standard analytical equipment, mostly chromatography
(HPLCs, gas chromatography or capillary electrophoresis). These technologies
represent important and versatile tools on which measurement methods for an
infinite range of molecules can be developed. They offer sensitivity, selectivity and
reliability, however they are expensive, slow and labor demanding. For instance,
HPLC monitoring of two different analytes in a microbioreactor would require two
different methods and potentially different columns or even detectors, with most
methods taking 10 to 20 minutes per sample that easily requires a few hundred
microliters (more than the full volume of some microbioreactors) [87]. Due to the
amount of effort, time, resources and limited amount of samples that can be
withdrawn from a microbioreactor for measurements with standard analytical
equipment, only a limited amount of data can be extracted.

1.5.3 Online analytics using integrated biosensors

The standard analytical equipment can only offer a limited amount of data on
processes in microbioreactors due to restrictively large sample amounts and
resources required for the analysis. Biosensors can be miniaturized as electrodes
or sensor spots for electrochemical and optical sensors, respectively, they can be
fabricated on sub-millimeter scale using biocompatible materials. As biosensors
lend themselves to miniaturization and have simplistic modes of operation, their
integration into microbioreactors would enable simplified analytics. Furthermore,
since biosensors can operate in a continuous mode, measurements can be done in
real time, not only providing rich data for the research but also serving as a tool for
the bioprocess control.

The integration of biosensors into microfluidics is not uncommon as it is
practiced in micro total analysis systems, e.g. clinical diagnostics [13]. Such chips
are designed around and optimized for the biosensing process only, whilst
microbioreactors, on the other hand, are exclusive for the biochemical reactions. As biosensors have to be integrated into microbioreactors noninvasively, they would have to work in relatively harsh bioprocess conditions. Integrated chips would thus require certain novel technical solutions, making the integration of biosensors into microbioreactors uncommon; a search in Science Direct and PubMed engines yields no conclusive results for the search words “microbioreactor”, “biosensor” and “process control” as of the time of writing. Hence, the integration of biosensors into microbioreactors could be considered as a novelty.

An example of potentially successful and highly beneficial integration of a biosensor into a microbioreactor is glucose monitoring. Glucose is commonly used substrate in cultivation bioprocessing on large as well as micro-scale. Optimizing the glucose concentration in a growth medium is of crucial importance as most production microorganisms produce the desired secondary metabolites – biopharmaceuticals or their precursors – only when exposed to a narrow window of concentrations [7]. Oliveira et al. [88] report optimization of glucose concentration for cultivation of yeast in a microbioreactor by exposing the cells to a gradient of glucose within the microbioreactor. The glucose gradient is reached by diffusion of highly concentrated glucose on one side into a glucose-free medium on the other. Sampling for glucose monitoring in this case is difficult due to the small volumes and necessary intrusions into the system. The authors therefore added a dye for optical monitoring into the glucose stream and assume that the diffusivity of the dye equals to the diffusivity of glucose [88]. The assumption of equal diffusivities is wrong as the sizes and chemical properties of the two molecules do not have much in common. Glucose biosensor, the first reported and nowadays one of the most developed biosensors [89] is on the other hand widely available and could aid glucose monitoring in an accurate, online manner. Inspired by this niche, a greater part of this thesis deals with the integration of glucose biosensor into diverse microbioreactors for biopharmaceutical purposes.

The reason for sparsity of research within the field of integration of biosensors into microfluidics can be attributed to the fact that biosensors on one hand require mild and specialized conditions that rarely meet the bioprocess conditions on the other hand. Thus several challenges have to be addressed during the design of an integrated system:

1. **Biosensor stability.** A biosensor is only as stable as its most fragile component, the biological recognition element in this case. Despite the effort to increase
the biosensors’ stability, their robustness still cannot match mechanical or physical sensors. Hence, the stability of a biosensor is even more crucial and has to be investigated before being integrated into microbioreactors, knowing the durability before a recalibration or replacement is required.

2. Sterility. Bioprocessing requires exclusivity of a desired microorganism, cell line or other biochemical system (enzymes or an enzymatic cascade) for optimal bioprocessing – a sterile environment. Due to diverse growth rates, an infected bioreactor or microbioreactor would behave differently as intruding microorganisms consume valuable substrates, pose a risk of outgrowing the cells intended for a bioprocess or inhibit the cell growth or the production of the desired molecules. Sterilization is therefore essential for a microbioreactor. However, most biological recognition components coagulate or otherwise deform when exposed to heat, chemicals or other standard sterilization agents thus losing the crucial binding capability. Hence, different ways of performing or bypassing the sterilization of biosensors have to be found before their integration into microbioreactors.

3. Matrix effects. Well studied phenomenon of measurement disturbance by molecules that coexist with the targeted analyte. The matrix effect is studied commonly for clinical applications of biosensors where aiding microfluidic tools are applied for sample pretreatment to avoid the phenomenon [13]. Different matrices can either increase or decrease the sensitivity of a biosensor towards the target molecule.

4. Sensitivity. Most biosensors are designed to have a high sensitivity and a low limit of detection as they are used for monitoring of clinical, biological or environmental samples in which the concentrations of the analyte are very low. Enhancing a biosensor’s range is difficult as it is limited by the kinetic properties of the recognition molecules, e.g. saturation of enzymes or antibodies. In contrast, microbioreactors are engineered to enhance the productivity, meaning that the concentrations of both substrates and products are considerably higher – for instance, glucose is one of the most highly concentrated substances in blood circulation, where its normal blood levels are around 5 mM, while glucose in a biocultivation process easily exceeds 100 mM. The extension of the biosensor range or development of an aiding technology is thus of crucial importance for the successful integration of a biosensor into a microbioreactor chip.
1.6 Aims and Objectives

The aim of the presented research is the development of technical solutions that would allow the integration of biosensors into microbioreactors platforms and microfluidic chips for biopharmaceutical processing, thus resulting in enhanced, online integrated bioanalytic systems aimed to relieve the analytical bottleneck of manual labor and expensive equipment. Due to the small sample volumes that biosensors require, the analytics of small-volume microbioreactor systems is enabled as the volumes of samples required for standard analytical equipment are too large [87].

Microfluidic chips, actuation systems, like pumps and mixers, physical sensors and biosensors and their respective production technologies were developed to work in concert with each other, further advancing the parallelization and high throughput capabilities of microbioreactors by relieving the analytical bottleneck.

The set objectives to meet these aims are:

1. Development of physical sensors and biosensors. Biosensors for integration purposes were developed targeting glucose, lactate, pyruvate and galactose, accompanied by an optical density sensor for cell biomass.
2. Development of pumping and mixing in microfluidics. Actuation within a microbioreactor is of crucial importance for bioprocessing [8,29]. Technologies for mixing and pumping were developed for fluid actuation in a manner that is noninvasive to the housed bioprocess.
3. Development of biosensor techniques for a range of microbioreactor applications. Technical solutions were developed in order to surmount the integrative challenges listed in Chapter 1.5.3.
4. Development of novel biosensing technologies. Molecularly imprinted polymers were investigated as a potential recognition component for their superior stability and range of potential targets, surmounting challenges listed in Chapter 1.5.3.
2 Development of biosensors and sensors for bioprocess monitoring

2.1 Introduction

2.1.1 Electrochemical enzymatic biosensors

As introduced in Chapter 1.4, biosensors are integrated biological recognition element-transducer devices capable of quantifiable detection of target molecules [31]. Electrochemical biosensors translate analyte concentrations into an electrical signal [76]; electrochemical biosensors featured herein featured amperometric signals and belonged to the second generation of mediator based biosensors. Glucose, lactate, pyruvate and galactose were the targeted analytes with their respective oxidases utilized as biological recognition components. Prussian blue was the mediator of choice for glucose, lactate and galactose biosensors (adapted from Ricci et al. [90]) while Meldola blue was used for the pyruvate biosensor (adapted from Abayomi et al. [43]). Thus, featured biosensors were of second generation.

The detection principle is generally the same regardless of the immobilized enzyme and is depicted in Figure 2. Quintessentially, oxidase mediated oxidation causes occurrence of hydrogen peroxide (catalytic recognition event). The mediator consumes hydrogen peroxide and induces a measurable electrical current if appropriate potential is applied. The optimal potential is found by cyclic voltammetry whilst the quantifiable electrical current is dependent on the analyte concentration.

Described electrochemical biosensors were based on carbon screen printed electrodes with silver basis; all the components were immobilized by physical adsorption and entrapped by a polymer membrane (Nafion®). Sensitivity, linear range and other biosensor characteristics were optimized by manipulating the amounts of enzymes and membrane on the working electrode.
2.1.2 Determination of biosensor stability based on thermally accelerated ageing

As biosensors rely on a biological recognition component, they are prone to ageing as any other biological compound and regardless of biosensor application its commercial success is dependent on its stability [91]. Stability mostly refers to the shelf life stability in case of single use biosensors, specifying the time for which a biosensor can be stored until its sensitivity drops below the acceptable threshold (I). This data is insufficient for biosensors that are expected to work continuously for longer periods as the usage leads to quicker deterioration of sensitivity (I). Decrease of sensitivity is time, operation mode (single use, reuse or continuous use) and temperature dependent. As ageing is accelerated at elevated temperatures, Arrhenius [92] and Schaal [93] models have been developed relying on correlation between the rate of deterioration and temperature.
As the biosensors developed herein are intended for integration into microbioreactors within biopharmaceutical industry, it is of crucial importance to determine their stability in storage (shelf-life), when reused and especially when used continuously (operation modes). Deterioration of sensitivity with time was monitored within all three operation modes at different temperatures, aiming to establish a correlation that would yield a protocol of rapid determination of stability characteristics (I). Provided information would allow to determine for how long a biosensor can be stored, how many times it can be reused as well as when it has to be recalibrated or replaced when integrated into a microbioreactor.

2.1.3 Optical enzymatic biosensors

Optical spectrophotometric methods are mainstream in chemical analysis e.g. for HPLC detectors. In recent years, optical devices for detection of partial oxygen pressure in fluids became commercially available [14]. Ehgrartner et al. report testing palladium(II) and platinum(II) meso-tetra(4-fluorophenyl) tetrabenzo porphyrin (PdTPTBPF and PtTPTBPF, respectively) embedded into polystyrene-silicone rubber composite with favorable oxygen sensitivity results [94]. Ramon et al., on the other hand, report the utilization of coaxial electrospun nanofibre material with PdTFP as an oxygen sensitive dye in PMMA in the inner layer fibre and PolymBlend® for the outer protective layer (II). Electro-spun material enables stable but simple adsorption immobilization of enzymes onto it, making it a great basis for a biosensor (II). Oxygen dependent electrochemical biosensors belong to the first generation. Swapping electrochemical oxygen electrode for an optical oxygen detection systems simplifies integration into microfluidics and potentially into microbioreactors. Oxygen sensitive materials can simply be enclosed into the microfluidic system using adhesives with the possibility of performing detection readouts through transparent materials [54,94]. A glucose optical biosensor was developed by immobilizing glucose oxidase onto the electro-spun oxygen sensitive material. As the architecture is oxygen dependent, bare biosensors were compared to ones integrated into a PMMA microfluidic platform that limits oxygen diffusivity.

2.1.4 Optical density sensor

Cell biomass monitoring is of crucial importance for cultivation bioprocessing as productivity directly depends on the cell growth and concentration [79]. This is best
monitored via optical density (OD) measurements as the turbidity (light scattering) of media increases with the increase of biomass concentration. OD measurements are dimensionless and are done by illumination at 600 or 650 nm of wavelength and monitoring the transmittance of light in a certain path length (usually 1 cm).

2.2 Aims and objectives

The aim of this chapter is the development of sensors and biosensors for integration into a microbioreactor for biopharmaceutical process monitoring.

Glucose is the single most important analyte for biopharmaceutical cultivations and was joined with lactate, galactose and pyruvate for the development of fully characterized electrochemical biosensors. An optical glucose biosensor was developed and its self-standing functionality was compared to the version integrated into a custom microfluidic platform. An optical density sensor using a 650 nm monochromatic laser was developed and validated for the biomass growth monitoring.

2.3 Materials and methods

All materials were analytical grade and sourced from Sigma Aldrich (Germany) unless otherwise specified.

2.3.1 Electrochemical enzymatic biosensors

Essential materials for the fabrication of electrochemical enzymatic biosensors were phosphate buffered saline (PBS) 100 mM, pH 7.4 tablets, iron (III) chloride, glutaraldehyde, glucose oxidase (GOx) from *Aspergillus niger*, pyruvate oxidase, lactate oxidase, galactose oxidase, albumin from bovine serum (BSA), Nafion® 117, hydrochloric acid (Merck Millipore, Germany), potassium ferricyanide (Baker, Holland), D(+)-glucose anhydrous (VWR, Belgium)), galactose, lactose and pyruvate. Reverse osmosis (deionized) water was prepared using Elga system (Elga LabWater, UK). Experiments were conducted using 3 electrode screen printed electrodes (carbon working electrode (WE) was 3 mm in diameter, sliver reference (RE) and carbon counter electrode (CE)) purchased from ECOBIO lab (Florence, Italy) and PalmSens potentiostat (PalmSens, Netherlands).

The Prussian Blue (PB) was physically adsorbed onto the carbon surface of the WE following a modified protocol developed by Ricci et al. [49]. Briefly: PB 0.1
A M mixture in 10 mM aqueous hydrochloric solution was prepared from the mixture of potassium ferricyanide and ferric chloride in 1:1 molar ratio. 10 µL of PB was dispensed onto the working electrode, followed by a 15 minute incubation at room temperature. After that, PB was rinsed off, first using 10 mM hydrochloric solution followed by deionized water. Rinsed electrodes were then incubated at 100°C for one hour and were cooled down ready for further modification.

2.5 µL of 1 % (V/V) gluteraldehyde in aqueous solution was dispensed onto the surfaces of the PB modified working electrode. Followed by an incubation step of 20 minutes or until completely dry. A fresh enzymatic cocktail of oxidase enzyme, Nafion and bovine serum albumin (BSA) was prepared. For every 3 µL of enzyme cocktail solution consisted of 1 µL of 0.1 U/µL of an enzyme (glucose oxidase, lactate oxidase or galactose oxidase, depending on the target of the enzyme), 1 µL of 0.1 % (V/V) aqueous solution of Nafion 117® and 1 µL of 5 % (m/V) aqueous solution of bovine serum albumin (BSA). 3 µL of the enzymatic cocktail were deposited onto the working electrode, followed by an overnight incubation at 8°C at 40 % relative humidity.

For the pyruvate biosensor the PB modification was replaced by Meldola blue on the same electrodes [43]. First, 10 mM solution of MB has been prepared in ethanol, followed by 1:1 dilution in deionized water. Such protocol was followed to facilitate the insolubility of MB in water and inconvenient wettability characteristics of ethanol. Nafion 117® was added to the mixture (1 % (V/V) final concentration) to trap MB and prevent it from being washed away from the electrode. 1 µL of described mixture was dispensed onto the WE followed by a 5-minute incubation at room temperature, after which the electrode was ready for further modifications.

The final stage of modification consisted of dispensing 3 µL of enzymatic cocktail onto the working electrode; each 3 µL of enzymatic cocktail consisted of 0.25 µL of 0.6 % aqueous solution of Nafion, 0.25 µL of 5 % (m/V) solution of BSA, 1 µL of aqueous solution of 0.25 U/µL, 0.5 µL of 10 mM aqueous solution of MgCl2, 0.5 µL of 10 mM aqueous solution of thiamine pyrophosphate (TPP) and 0.5 µL of 10 mM aqueous solution of FAD. Deionized water was used for all aqueous solutions while the BSA served as a protective component for the enzyme and MgCl2, TPP and FAD as cofactors. After dispensing the mixture onto the working electrode, the electrodes were incubated at 8°C and 40 % relative humidity overnight.

Following the fabrication protocols, each electrochemical biosensor was initially tested in cyclic voltammetry mode (CV). Three scans have been made.
between -0.5 and 0.5 V at the scanning rate of 0.05 V/s and step of 0.01 V. CV tests were carried out in a 50 µL 0.1 M PBS buffer droplet covering all three electrodes (WE, CE and RE) of the biosensors. In that manner, peak potentials for later amperometrical measurements were found. Biosensors were then amperometrically tested at the found peak potentials in respective standards prepared in 0.1 M PBS (50 µL droplets on biosensors).

2.3.2 Determination of biosensor stability based on thermally accelerated ageing

Electrochemical glucose oxidase biosensors were used as model biosensors for the determination of ageing characteristics and were fabricated according to the protocol described in Chapter 2.3.1. Shelf life was determined by storing several glucose biosensors at 22°C, 40°C and 50°C and daily monitoring the signal deterioration when exposed to a standard concentration of glucose. Each sensor was disposed after testing. Reusability tests were performed following the same protocol but with each biosensor being reused throughout the study at 4, 20, 35, 45 and 55°C.

Continuous use stability was determined by continuously measuring a 20 mL glucose standard) at 20, 35, 45 and 55°C until no change in signal was observed. Signal deterioration for each test was assessed. All sensors were tested with 1.5 mM glucose solution in PBS.

2.3.3 An optical enzymatic biosensor

The electrospun oxygen sensitive coaxial fiber tissue produced by NanoMyP S.L. (Granda, Spain) consisted of PolymBlend® (10 % m/m) in dimethylformamide (DMF) for the outer fiber and PMMA (6 % m/m) with PdTFPP (1 % m/m) for the inner fiber.

The microfluidic chip for housing of the polymer membrane was produced in PMMA plates (2 mm thick) that were CO₂ laser cut and bonded together using matching CO₂ laser cut medical grade pressure sensitive tape (LG, South Korea). The same adhesive was used for the immobilization of the oxygen sensitive tissue. Positioning of the 8 sensing chambers mimicked the geometry of a 96 well microtiter plate to fit into a spectrophotometric reader (Varioskan® Flash) as shown in Figure 9. The overall length of a single microfluidic channel with an inlet,
sensing chamber and outlet, was 30 mm, width was 2.5 mm and depth 0.1 mm. The depth of the sensing chamber was 0.2 mm and its diameter was 7 mm.

After the assembly of the chip, glucose oxidase (Sigma Aldrich, Germany) was immobilized by adhesion; 10 µL of glucose oxidase solution (1 mg/mL) in PBS (Sigma Aldrich, Germany; 0.1 M, pH 7.4) was dispensed directly onto the circular piece of oxygen sensitive tissue (6 mm in diameter), followed by 15-minute incubation at room temperature and rinsing with 10 mL of water.

The measurements were performed by inserting the biosensing chip into the microtiter plate reader. Luminescence readouts were executed at 405 nm excitation and 668 nm emission wavelengths with 5 nm bandwidth, delayed for 200 µs and measured for 100 ms. Initially, the sensing performance of different amounts of glucose oxidase on the oxygen sensitive tissue has been tested, followed by the building of a calibration curve using the previously found optimal enzyme concentration.

2.3.4 Optical density biosensor

Optical density sensor (here on referred to as “ODsens”) was based on an Arduino® programmable microcontroller. The housing of ODsens was custom 3D printed (fused filament fabrication 3D printing of polylactic acid (PLA)) to house a monochromatic laser at 650 nm of wavelength and a photodiode for readouts.

2.4 Results and discussion

2.4.1 Electrochemical glucose oxidase biosensor

In order to find the optimal sensitivity and linear range of the electrochemical glucose oxidase sensor, different versions with different amounts of immobilized enzyme and amount of diffusion limiting membrane (Nafion®) were tested. The versions of glucose biosensors with 0.074, 0.10, 0.37 and 1.85 U were fabricated and characterized. As shown in above, the tested biosensors were based on had a current limit of 1 µA that was independent of the amount of immobilized enzyme. The versions with high amounts of glucose oxidase were found to reach the current limit sooner than the biosensors with less enzyme. Low amounts of enzyme were found to be preferable for the fabrication of electrochemical glucose oxidase biosensors due to an extended linear range.
The next step in optimization was to find an optimal amount of diffusion limiting membrane. Two versions were tested, both bearing 0.074 U of glucose oxidase, one with 1 µL (V/V) of 0.1 % Nafion® and the other with the same amount of 0.5 % Nafion®. The results are shown in Figure 3 below and the lower amount of the diffusion limiting membrane was found to be optimal.

![Graph showing relationship between sensitivity and amount of enzyme or Nafion](image)

Fig. 3. Optimization of electrochemical glucose oxidase biosensor. Up: dependence of sensitivity on the amount of immobilized enzyme. Down: dependence of sensitivity on the amount of Nafion on the biosensor.

Optimized electrochemical glucose biosensor was found to have a very low theoretical limit of detection at 0.006 mM with the resolution of 0.004 mM. The linear range was 0 to 2.5 mM (Figure 4), but could be extended to up to 4 mM if non-linear model was used.
The intra-sensor reproducibility using the same concentration with the same electrode was well within 4% whilst an inter-sensor reproducibility of different biosensors from the same batch varied up to 5.7%. Hysteresis was found to be around 10% in the worst-case scenario. Selectivity testing was conducted by exposing the glucose biosensor to 1.5 mM galactose and comparing the signal to the one at equal concentration of glucose. The sensitivity to galactose was found to be negligible, proving a very high selectivity of the developed glucose biosensor.

When the glucose biosensor was exposed to a step change in glucose concentration, the observed change in signal was instantaneous, however, it took the biosensor up to 58 s to reach 90% of the stable readout. Complete biosensor characteristics are listed in Table 1.
Table 1. Characteristics of electrochemical glucose oxidase biosensor

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical minimal detected concentration</td>
<td>0.006 mM</td>
</tr>
<tr>
<td>Resolution</td>
<td>0.004 mM</td>
</tr>
<tr>
<td>Linear range</td>
<td>0-3 mM ($R^2=0.9985$)</td>
</tr>
<tr>
<td>Response time</td>
<td>$T_{90}=58$ s</td>
</tr>
<tr>
<td>Intra-sensor reproducibility</td>
<td>4.0 %</td>
</tr>
<tr>
<td>Inter-sensor reproducibility</td>
<td>5.7 %</td>
</tr>
<tr>
<td>Selectivity to galactose</td>
<td>0.016 %</td>
</tr>
<tr>
<td>Hysteresy</td>
<td>&lt;10.8 %</td>
</tr>
</tbody>
</table>

2.4.2 Electrochemical lactate oxidase biosensor

The electrochemical lactate oxidase biosensor underwent the same optimization procedure as the glucose oxidase biosensor. The theoretical limit of detection was found to be 0.025 mM with the readout resolution of 0.05 mM. The linear range was 0 to 1.5 mM with the response times ($t_{90}$) of less than 23 s. The complete characterization of the lactate oxidase biosensor is shown in Table 2 and the calibration curve is shown in Figure 5.

Table 2. Characteristics of electrochemical lactate oxidase biosensor

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical minimal detected concentration</td>
<td>0.025 mM</td>
</tr>
<tr>
<td>Resolution</td>
<td>0.05 mM</td>
</tr>
<tr>
<td>Linear range</td>
<td>0-1.5 mM ($R^2=0.9835$)</td>
</tr>
<tr>
<td>Response time</td>
<td>$T_{90}=23$ s</td>
</tr>
<tr>
<td>Intra-sensor reproducibility</td>
<td>10.9 %</td>
</tr>
<tr>
<td>Inter-sensor reproducibility</td>
<td>0.2 %</td>
</tr>
<tr>
<td>Hysteresy</td>
<td>&lt;4.3 %</td>
</tr>
</tbody>
</table>
2.4.3 Electrochemical pyruvate oxidase biosensor

The pyruvate biosensor was developed following an adapted protocol by Abayomi et al. [43]. In contrast with glucose, lactate and galactose biosensors, the pyruvate biosensor required the co-immobilization of MgCl₂, TPP and FAD to function. A calibration curve (Figure 6) was built with linear range of 0 to 10 mM featuring relatively low currents compared to other biosensors. The biosensor was found to be unstable during the testing, it showed high hysteresis and poor reusability as a result of unpredictable washing out of the immobilized cofactors. Developed pyruvate biosensor would thus only be useful for single use rather than for bioprocess monitoring.
Fig. 6. Calibration curve of electrochemical pyruvate oxidase biosensor.

2.4.4 Determination of biosensor stability based on thermally accelerated ageing

Three types of experiments were conducted in order to characterize the stability of a model (glucose) biosensor: shelf-life (storage before single use), reusability (repeated storage and use) and continuous use ageing (I). During the shelf-life ageing experiment, a batch of the glucose biosensors was divided into three groups, each exposed to a different temperature. The deterioration of signal was found to be linear for each temperature group (Figure 7). The linear regression was determined for each temperature, a value that corresponded to the deterioration rate (percentage of original signal lost per day). The deterioration rates were then plotted against their corresponding temperatures, yielding a linear dependency.
The reusability and continuous use experimental data were treated in the same way as for the shelf-life ageing. The results of continuous use are shown in Figure 8, yielding the same linear dependency of deterioration rate and temperature. This dependency was found to be a pillar of a universally applicable model for the determination of ageing characteristics of biosensors, as it allows calculating an expected deterioration rate for any temperature by extrapolation or interpolation. Indeed, the elevated temperature was found to enhance the phenomenon of ageing and the developed model enables a rapid determination of shelf-life or continuous use stability of a biosensor.

The reusability experiments, however, yielded unreliable results due to the chaotic nature of damage that biosensors undergo during the storage. The findings suggested that reusing a biosensor, at least without a prior calibration, would be unreliable (I).
Fig. 8. Determination of continuous stability of glucose biosensor. Left: plotted degradation of signals at different temperatures; right: determined coefficients plotted against the temperature. \( k \) represents degradation coefficients at different temperatures (I, published by permission of Elsevier).

2.4.5 Glucose oxidase biosensor based on oxygen sensitive material

Fig. 9. Architecture of the microfluidic chip with the oxygen sensitive material for optical detection of glucose. a) scheme and b) photograph (II, published by permission of Elsevier).

The development of optical glucose oxidase biosensor was done in collaboration with the University of Granada and NanoMyp S.L. (Granada, Spain). An electro-spun oxygen sensitive material served as a transducer whilst glucose oxidase was immobilized as a biological recognition component. Initially, an optimal amount of enzyme immobilized onto the oxygen sensitive tissue to form a biosensor was investigated by changing its concentration in the immobilization mixture. The optimum was found to be at 1.0 mg/mL followed by the construction of the calibration curve using just the oxygen sensitive tissue with glucose oxidase.
(without the microfluidic chip, in a microtiter plate) (grey line in Figure 10). Next, the same calibration experiment was conducted inside the described microfluidic chip (black line in Figure 10).

The calibration curves of the bare and integrated optical glucose biosensor showed significant differences. The integrated version was found to have an improved sensitivity and range; the latter was linear between 1 and 25 g/L (6 to 140 mM). Besides the sensitivity, the established linear range is highly beneficial for the bioprocess monitoring as it matches most biocultivation conditions in biopharmaceutical industry [79].

The difference between the calibration curves for the bare and the integrated biosensor can be attributed to the changes of oxygen transfer. The whole system is oxygen dependent, measuring the decrease of oxygen as a result of enzymatic oxidation of the analyte. In case of the bare sensor, the consumption of oxygen by the immobilized glucose oxidase competes with the oxygen that easily diffuses from the surrounding atmosphere. In case of the microfluidically enclosed version, small sizes of channels and PMMA casing significantly limits (but does not omit)
the oxygen transfer due to slow diffusivity. Integration of developed optical glucose biosensor into a microfluidic platform is hence of crucial importance when intended for biopharmaceutical process monitoring.

2.4.6 Experimental development and measurement methodology of optical density sensor

Optical density sensor, ODsens, consisted of a monochromatic laser and a photodiode facing each other, connected to an Arduino® microcontroller. ODsens was designed for simple, contact free integration onto a microbioreactor of translucent or transparent walls (Figure 11). Striving towards the simplicity that would allow low cost fabrication, ODsens was built using a 3D printer using components that cost a fraction of a euro and a microcontroller that can be bought for less than 10 euros.

![Fig. 11. ODsens optical density sensor in a 3D printed housing (black) click-on, contact free integrated onto a translucent microbioreactor. A – 3D printed microbioreactor; B – 3D printed stand; c – click-on ODsens.](image-url)
Quintessentially, when the amount of light falling onto the photodiode changes, it responds with the change of its own resistivity. The microcontroller measures that by comparing the photodiode resistivity to the resistor standard; matching it to the complete system is of crucial importance in order to maximize the sensitivity of the ODsens.

The resistor standard was optimized iteratively. An optimal one would be distinguished by the high raw readouts when exposed to the laser light and low readouts when exposed to darkness. The findings are shown in Figure 12; a 1 MΩ resistor was found to have the greatest difference between the readouts in the laser light and darkness and was hence used for all applications of the ODsens.

![Graph](image)

Fig. 12. Experimental choice of resistor standards for ODsens. The optimum is visible at 1 MΩ.

ODsens was programmed to have an automatic calibration protocol that measured the raw signal in the darkness (when the laser was turned off), $S_{\text{min}}$, followed by the determination of the same signal when exposed to a flash of laser light, $S_{\text{max}}$. During the calibration protocol, the ODsens was exposed to a microorganism-free cultivation medium where optical density would later be measured.

The ODsens measurement protocol dictates the measurement of raw signal during a flash of laser light calculating the light transmittance $T$:
Optical density, OD, was calculated from the light transmittance as follows:

\[ OD = -\log_{10} T \]

In order to validate the performance of the ODsens, its response to set dye concentrations was independently compared to the results given using the Varioskan® microtiter plate reader. Two dilution series of green food dye were prepared taking two different final sample volumes of 150 and 50 µL and pipetting into a 96-well microtiter plate. The calibration curves were then compared after the signal normalization that was necessary as optical density a dimensionless parameter that cannot be directly compared between the instruments, but its normalized values should. The results are shown in Figure 13.

![Figure 13. Comparison of ODsens and Varioskan calibration curves in a microtiter plate using 50 and 150 µL of green dye dilution line as validation of ODsens performance (IV).](image)

The performance of ODsens was evaluated as satisfactory as the readouts corresponded well to the Varioskan® with coefficients of determination of 0.98 for both 50 µL and 150 µL dilution lines. Compared to the state of the art, the optical density sensor of similar architecture is well known [79]. The novelty of reported ODsens is in the simplification of components (monochromatic laser providing
appropriate light of appropriate wavelength rather than spectrophotometric analysis of wide bandwidth light sources), utilization of low cost microcontrollers allows cheap parallelization and 3D printed housing accounts for simple, click-on integration.

2.5 Conclusions

2.5.1 Electrochemical enzymatic biosensors

Electrochemical glucose oxidase, lactate oxidase and pyruvate oxidase biosensors have been developed and characterized. All developed electrochemical biosensors featured the expected sensitivity, linear range, limit of detection and other characteristics. The stability of glucose oxidase and lactate oxidase biosensors is sufficient for the integration into microbioreactors and can be expected to perform online, real time monitoring. The linear ranges of both glucose and lactate biosensors do not meet the concentrations to be monitored within microbioreactors due to the limitations of enzyme and electrode saturation. Further technical solutions will be required to facilitate integration.

Pyruvate and galactose biosensors, on the other hand, proved unstable due to the washing out of the immobilized components (mediators and cofactors) that caused the loss of signal when exposed to several samples. These biosensors worked only as single-use and could not be considered for integration into microbioreactors.

2.5.2 Determination of biosensor stability based on thermally accelerated ageing

Shelf-life, reusability and continuous use tests proved the dependency of ageing on temperature and handling. During the continuous measurements, the model glucose biosensors aged more rapidly than the ones in storage; reusability of biosensors proved unpredictable due to the random physical damage encountered during the handling.

The biosensors’ deterioration rate was found to be linearly dependent on temperature, enabling the prediction of a long-term stability at low temperatures by rapid tests at elevated temperatures. Besides yielding invaluable information on the model electrochemical glucose biosensor, the protocol of determination of
biosensor stability based on thermally accelerated ageing was found to be transferrable to all biosensors.

2.5.3 Optical enzymatic biosensor

The oxygen sensitive material utilized for the development of optical glucose biosensor proved to exhibit promising properties for the integration into microbioreactors due to the simplicity of integration, contactless optical monitoring and versatility. The initial sensitivity of the bare biosensing material with integrated glucose oxidase was poor, however, its integration into a special microfluidic chip enhanced the sensitivity in high range, a favorable feature for bioprocess monitoring where glucose is highly concentrated. The developed optical biosensor detects glucose via consumption of oxygen by glucose oxidase. The sensitivity is hence dependent on the oxygen transfer that was limited when integrated into a microfluidic platform, thus increasing the sensitivity of a bare system.

2.5.4 Optical density sensor

Optical density is a standard monitored parameter within the bioprocess control. The developed optical density sensor, ODsens, proved to have an appropriate, validated sensitivity, whilst offering simplified integration (click-on). Detection is contact-free and nonintrusive through the translucent or transparent walls due to the calibration function, reducing the probability of contamination as no holes are required. Utilization of monochromatic laser relieves the necessity for an expensive optical spectrophotometer. The inexpensive electronic components were integrated into a 3D printed housing, reducing the price of the whole system and enabling further parallelization.
3 Development of microfluidic fabrication technologies and actuators for biopharmaceutical bioprocessing

3.1 Introduction

3.1.1 Fabrication technologies of microfluidic chips and microbioreactors

Microfluidic and microbioreactor technology is enabled by micro-fabrication tools that allow the production of sub-micrometer features [15]. Although the architectures and purposes of microfluidic chips and microbioreactors vary, the fabrication techniques are common and can be divided into molding, abrasive and additive technologies. Materials for microfluidics and microbioreactors have to be biocompatible with the intended bioprocesses and must have favorable physical features (rigidity or flexibility, transparency or translucency, oxygen diffusivity etc.) e.g. for the integration of sensors and biosensors.

Polydimethylsiloxane (PDMS) is a biocompatible, optically transparent, inexpensive silicone elastomer often used for fabrication of microfluidic devices [95]. PDMS chips are usually fabricated through a soft lithography process [95] that allows rapid reproduction of the same structure, however, it requires an effort for the development of a mold. The utilization of laser cutters (e.g. CO2-laser cutter) allows for rapid prototyping of microfluidic chips due to the key advantages of time and cost effectiveness, precision and simple control [96]. Commonly, polymethyl methacrylate (PMMA) is used as a substrate for laser cut microfluidic chips as it can be etched by the laser light whilst being biocompatible, optically translucent and inexpensive [96]. The major disadvantage of laser cutting is poor control over ablation that has to be surmounted for accurate and reproducible fabrication [96].

Laser cutter is used to fabricate necessary micro-channels and other features on the surface of a PMMA plate which has to be bonded to another one in order to seal the microfluidic chip. Due to the thermoplastic features of PMMA, it can be thermally bonded by exposing it to elevated temperatures, softening the polymer chains on the surface, followed by mechanically pressing the two plates together that causes the softened polymer chains to bond. Recently, Yu et al. reported that thermal bonding of thermoplastics including PMMA can be improved by prior plasma treatment that also enhances the wettability of the final microfluidic chip.
Solvents like ethanol can also be used to soften up the polymer chains on the surfaces of thermoplastics, allowing consecutive bonding when mechanically pressed together [98].

3D printing is an additive fabrication technology, using computer guidance to build three dimensional products in layers [99]. Recent advances in the availability and precision of 3D printing enabled this technology to be used for fabrication of microbioreactors and microfluidic chips. The main advantages of 3D printing are complete automation, complexity of the final products as well as cost and time effectiveness. Several distinctive 3D printing technologies are commercially available; the most widespread fused deposition modeling (FDM) also known as fused filament fabrication (FFF), stereolithography (SLA) and selective laser sintering (SLS) [20]. The FDM/FFF technology relies on a three dimensionally guided heated nozzle to melt the raw material (usually in a form of a filament), followed by solidification at the exit of the nozzle, forming a layered product. SLA technology uses a monomer resin as a raw material that is polymerized by an ultraviolet laser, causing 3D guided solidification in layers, forming the desired object. Similar to the SLA, the SLS technology uses a laser to solidify raw material in a form of a powder into a desired product.

3D printing technologies vary in price of instrumentation and raw materials, precision and fabrication speed. The FDM/FFF technology is the cheapest and uses inexpensive thermoplastics, finishing the product rapidly but at a cost of a relatively low resolution of approximately 0.5 mm. SLA and SLS 3D printing technologies offer a superior, micrometer resolution at a significantly greater expense and lower speed.

3D printing has been reported as a promising technology for fabrication of microfluidic devices since it is cheap and enables a fully automated building of closed, one piece, complex microfluidic chips [20]. Utilization of 3D printing for microfluidics is common; Hwang et al. for instance report the fabrication of 3D printed molds for casting microfluidic PDMS chips [100] whilst Gowers et al. report 3D printing of a complete microfluidic chip with integrated glucose and lactate electrochemical biosensors for the analysis of subcutaneous microdialysate in cyclists undergoing a training regime [101].

Microbioreactors and microfluidics strive towards low cost, miniaturization and parallelization, hence affordable, yet precise technologies for a rapid prototyping were investigated herein: laser cutting of PMMA using different bonding techniques, FDM/FFF and SLA 3D printing.
3.1.2 Actuation within microfluidics

In order for a microfluidic chip to facilitate lab functions, pumping and mixing functions are of crucial importance [102] as they assure the delivery of substrates, the removal of products and the homogenization of process fluids. All integrated functions, systems and materials have to be compatible with the housed bioprocess, reassuring that no interference (e.g. binding of products onto the walls of microfluidic chips or infection of fermentation broth through the mixing shaft seals) occurs.

Microfluidic mixing, in contrast to large scale mixing, is laminar and governed by diffusive processes; most microfluidic mixers are hence designed to enhance the mass transfer by reducing the diffusion lengths and enlarging the contact surfaces between the fluids that are intended to be mixed [16]. Microfluidic mixers can be either passive, relying on the kinetic energy of fluids, or active where the energy for mixing is provided externally. Passive mixers are diverse, divided into lamination-based and chaotic advection-based mixing. The first group relies on the segmentation of flow through the inertia effects whilst the latter mixes the component fluids by splitting, rearranging and combining the flows [103]. The most common chaotic advection-based solutions include intersecting channels, convergent-divergent channels, three dimensional structures, embedded barriers, staggered herringbone structures, twisted channels and Tesla structures [103].

Active mixing requires external energy input; Jo et al. report using an acoustic wave to facilitate mixing [104] whilst Shang et al. used a piezoelectric disk to induce a vortex that greatly enhanced mixing [105]. Insertion of magnetic beads and the exposure of the microfluidic chip to a rotating magnetic field induce effective, short distance and noninvasive mixing [106]. The passive staggered herringbone and the active magnetic mixers were tested and developed herein.

Pumping technologies can be external (e.g. syringe or peristaltic pumps) or internal. Internal microfluidic pump can either be a miniaturized large-scale technology (e.g. impeller, gear or centrifugal pump) or specific for the microfluidic applications. Wang et al. report using a micro-blower microfluidic pump [107] that is reminiscent of large scale air pressure pumps whilst Xia et al. used an electro-active polymer to construct a microfluidic pump [108] that is unlikely to work on a large scale. Herein, impeller, gear and centrifugal pumps have been investigated. Magnetic actuation was used throughout as it uniquely provides a contact-free momentum for fluid propulsion, allowing simple integration and complete sealing of the chip containing the mixer.
3.2 Aims and objectives

The aim of this chapter is to research the fabrication and actuation techniques that would later facilitate the development of microbioreactors and microfluidic chips for biopharmaceutical processing.

The PMMA laser cutting technology was investigated for fabrication of microfluidic chips. Thermal, solvent and adhesive bonding techniques were tested and a technology that provided simple, cheap and leakage-free bonding was established for later research.

3D printing was investigated as a novel technique for fabrication of microbioreactors, investigating different methodologies and materials suitable for sensor and biosensor integration, in particular, transparency for contact-free integration of optical density sensor.

All developed mixing systems were tested by monitoring mixing of glucose solution and blank buffer using an integrated electrochemical biosensor (developed within the scope of Chapter 2). Microfluidic pumping systems were evaluated by monitoring the flow rates across the working range.

3.3 Materials and methods

3.3.1 PMMA microfluidic chip fabrication

A CO$_2$-laser cutter (Jinan King Rabbit, China) was used to cut and engrave microfluidic features into PMMA (Perspex, UK). The PMMA plates were pressed together using a heat press (generic, China). Chloroform and 95 % (V/V) ethanol for surface treatment were sourced from Sigma Aldrich, Germany, and the double sided 100 µm thick medical grade adhesive from Adhesive Research, Ireland, or LG, South Korea (equal specifications and performances).

In case of heat bonding tests, the PMMA chips were individually preheated in an oven at 80°C before being pressed together in a heat press at a range of tested temperatures. In case of solvent facilitated bonding, the surfaces of the PMMA chips were exposed to the chloroform or ethanol vapor before being pressed together. Different exposure times were tested. The microfluidic chips that have undergone thermal or solvent bonding had their micro-channels and features engraved into one of the PMMA plates. The adhesive bonded microfluidic chips had channels engraved directly into the adhesive tape, however more complex features (e.g. herringbone mixer) were engraved into one of the PMMA plates.
3.3.2 3D printing for fabrication of microfluidic chips and microbioreactors

FFF (DaVinci 2.0 Duo) and SLA 3D printers (Nobel 1.0) were acquired from XYZprinting, Taiwan. Transparent resin from XYZprinting was used for the SLA 3D printer whilst the polylactic acid (PLA) and the PMMA filaments were sourced from 3D Prima, Sweden and FilamentWorld, Germany, respectively.

3.3.3 Development and evaluation of passive mixing

The passive mixers were fabricated following the developed CO₂-laser cutting techniques in PMMA and using a double sided medical grade adhesive (LG, South Korea) for bonding.

Glucose (Sigma Aldrich, Germany) was used as a tracer in the phosphate buffered saline at 0.1 M (Sigma Aldrich, Germany), that was mixed in developed mixers with the blank buffer. Two New Era NE-1000 programmable syringe pumps (New Era Pump Systems, NY, USA) were used for pumping. Glucose was monitored at the outlet was monitored using electrochemical glucose sensors fabricated according to the protocol described in Chapter 2.3.1. Blue food dye (DrOetker, Germany) was used for visual inspection.

3.3.4 Development and evaluation of active mixing

The active mixing was developed in microfluidic chips made by CO₂-laser cutting (Jinan King Rabbit, China) into PMMA (Perspex, UK). 2 mm magnetic balls (VWT, Finland) were placed into a 3 mm deep, 5 mm in diameter chamber in as the adhesive (LG, South Korea) bonded PMMA chips. 12 V brushed electric motors with reduction gearbox (generic, China) were used for rotating the magnetic wheel that was laser cut into PMMA with inserted magnets. An FFF 3D printed motor holder also served as a holder for the PMMA chip.

Active mixing was evaluated following the same protocol as described for the passive mixing.

3.3.5 Development and evaluation of a microfluidic pump

The microfluidic pumps were made from the CO₂-laser cut (Jinan King Rabbit, China) PMMA plates (Perspex, UK) and bonded using the double sided medical
grade adhesive (LG, South Korea) in different geometries. A cylindrical magnetic stirrer (2 mm long and 1 mm in diameter, VWT, Finland) was inserted into the chamber of 3 mm in diameter and 2.5 mm in depth. The rotating magnetic field was provided by the same system as described for active mixing. The flow rates were measured using a stopwatch and a laboratory scale.

3.4 Results and discussion

3.4.1 PMMA microfluidic chip fabrication

A CO₂-laser cutter offers several advantages when used for fabrication of microfluidic chips including speed and precision, however the latter is only planar. The depth of an engraving depends on the laser beam strength and material properties, thus appropriate settings for a desired depth have to be found empirically. For our purposes, the CO₂-laser cutter was always set to 3.5 µA that was found to reproducibly cause a 100 µm deep channel in the transparent PMMA sourced from Perspex, UK. The setting was found by counting the number of repetitions which were required by the device to cut 1, 2 and 2.5 mm thick PMMA plates (10, 20 or 25 cuts, respectively).

The engraving of the desired features into the PMMA plates was followed by an investigation of bonding techniques:

- Bonding using ethanol, heat and pressure. The engraved PMMA plates were exposed to 95 % (V/V) ethanol for up to 12 hours, followed by the alignment and compression in a heat press at the temperatures of up to 50°C (higher temperatures caused severe deformations of the plates). The methodology yielded weak bonds that did not seal the microfluidic chips.

- Bonding using chloroform and pressure. The engraved PMMA plates were exposed sticky face down to the chloroform vapor for up to 2 minutes in a closed petri dish, where a chip was positioned on the small glass pillars to lift the PMMA plates around 3 mm away from the surface of the liquid. Immediately after the exposure to chloroform, the plates were aligned and pressed together manually. Chloroform was found to substantially soften the exposed faces of PMMA. While the bond was strong and permanent, the produced microfluidic chips were prone to leakage due to the uneven effects of chloroform vapor over the surface of the PMMA plate. Furthermore, as chloroform softens up the surfaces of the PMMA plates, the geometry engraved
into the plates was found to severely deform when the layers were pressed together.

- Bonding using a double-sided pressure sensitive medical grade adhesive. This method required laser cutting of an additional layer for the microfluidic chip into the adhesive. As the thickness of the used adhesive used was known (100 µm from both suppliers), the microfluidic channels were cut only into the adhesive in order to keep the volume down where necessary. This methodology was found to be by far the most user friendly, yielding reliably and reproducibly sealed microfluidic chips. The created chips could last for week-long continuous processing, however, substantially longer times and strong solvents would cause the swelling of the adhesive followed by the failure of the chip.

The developed methodology of microfluidic chip fabrication was comparable to the reported state of the art chips as it allowed a reliable and accurate building of microfluidics that was necessary for the novel research in the field of integration of biosensors into microbioreactors for biopharmaceutical processing.

### 3.4.2 3D printing for fabrication of microfluidic chips and microbioreactors

During the time of writing, the 3D printing technology was found to be pioneered as a fabrication tool for microfluidic chips [20,100,101], however a search with ScienceDirect engine using “3D printed microbioreactor” as keywords yielded no-third party hits, suggesting the limited use of 3D printing for microbioreactor fabrication.

FFF and SLA 3D printing technologies were tested for the fabrication of a 1 mL microbioreactor (Figure 14) with two inlet/outlet channels (0.5 mm in diameter).
The PMMA transparent filament was used as a raw material for the FFF 3D printer. The main advantage of this technology was the speed and the low price of fabrication. The resolution of the FFF 3D printer was found to be largely limited by the nozzle diameter (400 µm) and was not capable to produce microfluidic channels of less than 1 mm in diameter. Due to the layer fabrication approach, long, unsupported (bridge-like) features tended to deform downwards. That caused the ceiling of the produced microbioreactor to be slightly curved inwards. The surface roughness of the final product caused the walls to be translucent rather than transparent; the light scattering would not allow the appropriate integration of the click-on optical density sensor.

The SLA 3D printing of the same microbioreactor yielded an improvement. More than a 10-fold slower process fabricated a product with a 25 µm precision suitable also for sub-millimeter micro-channels. The SLA 3D printed micro-channels were found to be of limited length; despite the fact that the fabrication of channels smaller than 100 µm was found to be possible, the highly viscous resin proved to be difficult to clear from the channels rendering them useless. 20 mm long and 0.5 mm wide channels caused no problems; additionally, the final product was highly translucent and could be easily made transparent by simplistic surface
treatment (e.g. brushing), making the integration of the click-on optical density sensor possible.

The SLA 3D printing was found unsuitable for production of high precision microfluidic chips mostly due to the required channel lengths. However, the successful fabrication of a microbioreactor was however novel and the device was later used for cultivation of yeast (reported in Chapter 4).

### 3.4.3 Development and evaluation of passive mixing

The purpose of passive mixing is the homogenization of the incoming liquids using no external power input but the kinetic energy of the fluid. Due to the micro-scale channels and features, the staggered “herringbone”-like structure was used to induce splitting, rearranging and combining the component fluids and thus aiding the mass transfer by diffusion [103]. The herringbone mixer was 50 mm long, 2 mm wide with depth between 100 and 200 µm due to the engraved structure (an example of integration of the same herringbone passive mixer is shown in Figure 15).

![Fig. 15. An example of a microfluidic chip with integrated herringbone passive mixer. A) Integrated herringbone mixer, B) enlarged herringbone pattern, C) serpentine structure to allow reaction time.](image-url)
The tested mixer consisted of two inlets, one connected to a syringe pump with glucose solution in PBS and the other to another syringe pump with the buffer only, and herringbone passive mixer that directly led to an outlet. By varying the flow rates from the two syringe pumps, the different glucose concentrations were expected at the outlet (Figure 16). As the readout values were stable and consistent with expectations, the herringbone passive mixer proved to have appropriately homogenized the mixture.

The developed passive mixer was not a novelty, however, it added to the microfluidic process unit operations that facilitated modularly building of more complex microfluidic systems for integration of biosensors into microbioreactors.

![Fig. 16. Evaluation of passive mixing of glucose solution and buffer using electrochemical glucose oxidase biosensor. According to known incoming concentrations and flow rates, expected concentrations have been calculated and listed near corresponding plateaus in the figure. 4 mM concentration was beyond the linear range of the glucose biosensor (III, published by permission of Elsevier).](image)

### 3.4.4 Development and evaluation of active mixing

An active microfluidic mixer uses the external energy input for homogenization of fluids in a microfluidic chip. A special magnetic actuation was developed for the
purpose: compared to using mechanically complicated shaft for transfer of momentum that would require the use of bearings and seals prone to leakage or infect the mixing chamber, magnetic mixing was contactless (III). The two magnetic balls rotated when exposed to a rotating magnetic field powered by a magnetic wheel on an electric motor.

A microfluidic flow separator was added downstream from the mixing chamber. In contrast to the passive mixing that cannot be turned off, the active mixing can. When the mixing is turned off, unequal tracer (glucose) concentrations are expected to be found at the outlets. Hence, the separator served as a negative probe when the mixing was turned off.

Fig. 17. Photograph of the active mixing evaluation experiment. An active mixer was coupled with a flow separator where glucose solution and blue dye have been used at 2x100 µL/min to monitor the phenomenon. The left hand side of the picture shows negative control where active mixing was turned off and the right hand side features the mixing turned on (III, published by permission of Elsevier).

The initial tests of active mixing using blue food dye, as shown in Figure 17, demonstrated a great difference in color between the two channels after the separator when the mixing was turned off. On the contrary, when the active mixing was turned on it facilitated the same colors between the channels. In order to analyze the efficiency, the experiment was repeated using glucose as a tracer (5 mM in PBS) monitored in one of the channels downstream from the separator at the combined total flow rates of 100, 200 and 400 µL/min (Figure 18). By switching
the mixing first off and then on, a negative control could be compared to the mixing operation (III).

Fig. 18. Evaluation of active mixing coupled with a microfluidic separator. The flow rates were set at 2x50, 2x100 and 2x200 µL/min for 0-400, 400-800 and 800-1200 s periods, respectively. For the first 200s of each period the mixing was turned off and for the other 200 on. 0 mM glucose readout would mean no mixing whilst 2.5 mM readout means complete homogenization (III, published by permission of Elsevier).

The efficiency of active mixing was best at low flow rates; it remained stable at 100 % efficiency (2.5 mM readout corresponded to complete homogenization) at 100 µL/min, but dropped to 95 % at 200 and 400 µL/min. The reason for the decreased efficiency at higher flow rates was that lower residence times within the mixing chamber did not allow for complete homogenization. The high residence times also affected the negative controls as at lower flow rates the small diameter of the microfluidic channels enabled the diffusive homogenization.

The developed active mixing system was easy to use since the microfluidic chip was simply clicked onto the holder with a magnetic wheel. The active mixing facilitated high efficiencies in a small amount of space compared to rather large mixing structures that are required for the passive mixing. Furthermore, the active
mixing was found advantageous over the passive one as it could work in batch, when the flows were stopped, making it suitable for stirring within microbioreactors. Consequently, the developed magnetic mixing was used for the cultivation in the microbioreactor described in Chapter 4.

3.4.5 Development and evaluation of a microfluidic pump

The microfluidic pumping is essential for the propulsion of liquids through a chip and is required for a substantial pressure drop that is caused by small dimensions of channels. Attempts were made to miniaturize several existing large-scale pumping systems: a gear pump, an impeller pump and a centrifugal pump. All systems were using the same magnetic actuation system as described for active mixing for the same advantages (simplicity and leakage free contactless actuation). The gear and impeller pump proved difficult to fabricate and required high torque to be moved due to tight tolerances necessary for pumping. To surmount the problem, the torque was increased by using larger diameters of the gears or impeller, however, such solutions had an internal volume which was too large to be integrated into microfluidics.

The micro-centrifugal pump (Figure 19) however, operated at higher speeds and lower torques that required considerably lower internal volumes (III). A magnetic stirring bar (1 mm in diameter and 2 mm long) was placed into a 3 mm wide circular

Fig. 19. Left: Photo of the microfluidic centrifugal pump. A – holder with a magnetic wheel powered by an electromotor; B – magnetic stirrer inside the pumping chamber with the inlet at the top; C – outlet; D – power supply (III). Right: Scheme of microfluidic centrifugal pump. Top: top view; bottom: side view. The pressure gradient is shown in white as low pressure and in orange as high pressure that is caused by spinning of the magnetic stirrer depicted in grey.
pumping chamber with a radial inlet and tangential outlet as shown in Figure 19. Quick spinning of the magnetic stirrer caused a centrifugation effect, forcing the liquid towards the walls where the pressure was increased while decreasing the pressure in the center. Positioning of the inlet in the spot with low pressure and the outlet tangentially streamlined at high pressure point caused the pumping effect.

Characterization of microfluidic centrifugal pump was executed by varying the speed of electric motor via the applied potential (III). The flow rate was measured by timing and weighing the pumped fluid and the results are shown in Figure 20.

![Fig. 20. Characterization of pumping performance at different potentials/motor speeds. The black line with circles depicts pumping flow rate whilst the grey line with triangles corresponds to current consumption (III, published by permission of Elsevier).](image)

The pump was found to have a maximum flow rate of 370 µL/min and a relatively narrow window of operation in regard to the applied potential. A minimum of 780 mV had to be applied in order to spin the magnetic stirrer with a sufficient speed to surpass the flow resistance within the chip. The window of operation was closed by the decoupling of the magnetic wheel and the magnetic stirrer; at high revolutions rate, the torque which was required for spinning the magnet surpassed the maximum torque between the magnetic stirrer and the magnetic wheel. Small size, simplicity and sufficient flow rates make the innovative micro-centrifugal pump applicable for actuation in microfluidic devices.
3.5 Conclusions

The developed microfluidic process unit operations combined with the fabrication techniques combined form a microfluidic toolkit that is essential for further developments of microbioreactor and microfluidic systems for the biopharmaceutical process control. Methodologies and technologies were later used modularly in complex applications, e.g. the SLA 3D printing of microbioreactor housing, laser cutting of a PMMA biosensor integration chip for the same microbioreactor that also featured active mixing as described in Chapter 4.

The described toolkit including the modular chips and the unit operations were developed and adapted to fit the micro-scale pharmaceutical bioprocessing; all components were biocompatible, sterilizable and suitable for the integration of biosensors and microbioreactors. Besides serving as a toolkit of essential microfluidic modules for later advancements, the utilization of 3D printing for fabrication of a microbioreactor, an active mixer coupled with a separator for negative control and micro-centrifugal pump were novel and original.
4 Integrated on-chip online analytics for biopharmaceutical process monitoring

4.1 Introduction

Biopharmaceutical process monitoring is of crucial importance for maintaining optimal bioprocess conditions that lead to optimum efficiency and yields [79]. Physical parameters (temperature, pH, partial oxygen pressure etc.) in microbioreactors are monitored by integrated sensors [14]. However, the monitoring of product yields and substrate consumption remains in the domain of standard chemical analytical equipment like HPLCs and GCs. The analysis is therefore time, labor and equipment intensive and it requires large samples; the volumes of which easily exceed the total volume of a microbioreactor. Analytics of small volumes hence remains a bottleneck in the development of inexpensive, parallelizable and high throughput microbioreactors for biopharmaceutical processing.

The integration of biosensors into microbioreactors can surpass the challenges since they are cheap, simple, offer real time measurements and lend themselves to miniaturization. Several challenges for the integration of biosensors into microbioreactor have been listed in Chapter 1.5.3: biosensor stability, sterility, matrix effects and sensitivity. Before the integration of a biosensor into a microbioreactor, those challenges have to be assessed and overcome in order for biosensors to provide reliable data without disturbing the bioprocess in a microbioreactor (IV).

4.1.1 Residence time distribution monitoring using glucose as a tracer in a packed bed microbioreactor

Bajic et al. [26] developed a line of packed bed microbioreactors of different lengths and widths with immobilized Novozym® 435 transketolaze particles. In order to fully understand and characterize the bioprocess, residence time distribution (RTD) inside the packed bed reactors had to be checked. Glucose was chosen as an inert tracer. Due to the small volumes of the microbioreactors, sampling and detection with standard analytical equipment was impossible. Lali et al. report RTD in a large scale cylindrical metal foam reactor often used for hydrogenation reactions [109]. The RTD study was conducted using the injection
of ethanol at the inlet and detection of refractive index at the outlet using a flow cell. The combination of glucose and glucose biosensor for RTD monitoring of packed bed reactors was expected to yield more reliable results of higher resolution due to the sensitivity of the integrated biosensor and small internal volume of the microfluidic flow cell where the sensor was integrated.

The integration of the glucose biosensor was direct using a 4 µL flow cell as the fluid downstream from the packed bed microbioreactor did not require sterility. As the RTD experiments were independent of temperature, it was kept down in order to aid the stability of the integrated biosensor. Furthermore, the choice of buffer and glucose concentrations would not affect the results, hence an appropriate concentration of glucose was injected into the system for the outlet concentrations to be within the range of the biosensor.

4.1.2 Online monitoring of lactose hydrolysis in a packed bed microbioreactor

65 % of the world population suffers from a reduced ability to digest lactose after infancy [110]. Dairy industry therefore offers products with lactose reduced or cleared. Besides utilization of lactic bacteria for the consumption of lactose, several enzymatic products have been developed for the purpose. Lactase (beta-galactosidase) is an enzyme that consumes lactose and hydrolyses it into easily digestible glucose and galactose.

Lentikat’s® developed polyvinyl alcohol beads with immobilized lactase for the purpose that were tested herein. In order to simplify the characterization of flow processing of lactose, the beads were packed into a packed-bed microbioreactor. The efficiency of lactose hydrolysis was monitored via glucose concentrations; according to the stoichiometry of the biotransformation, for each mole of consumed lactose one mole of glucose should be yielded. An HPLC was used as a reference method, however it required large samples and the measurements were time consuming. In order to simplify the process, a glucose biosensor was integrated downstream from the microbioreactor in order to relieve the analytical bottleneck.

The integration of the glucose biosensor was direct using a 4 µL flow cell as the fluid downstream from the packed bed microbioreactor did not require sterility. The bioprocess buffer was found suitable for the biosensor as it did not cause matrix effects. Room temperatures suited the biosensor to work continuously for up to a whole day whilst a buffer injection system was placed between the packed bed
reactor and the biosensor integration chip was placed to dilute the product in order for its concentration to match the sensitivity of the glucose biosensor.

4.1.3 Cultivation in a microbioreactor with integrated glucose biosensor and optical density biosensor

Cell cultivation is of critical importance in the biopharmaceutical industry and is used for pre-clinical (e.g. drug screening and toxicity tests) and biopharmaceutical production research (e.g. strain or growth medium optimization) as well as for the production of final bioharmaceuticals [7].

An SLA 3D printed microbioreactor developed within Chapter 3 was used for cultivation of a model microorganism, *Saccharomyces cerevisiae*. The microbioreactor, nicknamed “Milireactor” for its 1 mL internal cultivation volume, featured magnetic mixing (as described in Chapter 3.4.4) and an optical density sensor (ODsens, described in Chapter 2.4.6) (IV). As glucose is a single most widespread nutrient for microorganisms and is frequently used for cultivations, a glucose biosensor was chosen as a model biosensor for the integration into the Milireactor.

Sterility, matrix effects and sensitivity challenges had to be addressed in order to develop a fully functional microbioreactor system with an integrated glucose biosensor. The microbioreactor featured a biocultivation process that required sterility. However, the sterilization of glucose biosensor would cause its inactivation. The bioprocess fermentation broth would cause unpredictable matrix effects and the glucose concentrations within reach up to 20 g/L. Therefore, an integration chip with a diffusion limiting membrane and internal buffer flow was developed to surmount all the challenges of the biosensor integration (biosensor stability, sterility, matrix effects and sensitivity). Sterility was addressed by the fact that the membrane would prevent contamination of the sterile microbioreactor environment from non-sterile buffer flow within the biosensor integration chip. Therefore, the integration chip was placed first into the microbioreactor and both were heat sterilized, followed by non-sterile integration of the glucose biosensor onto the integration chip. The membrane had the second function of limiting the diffusion of glucose into the buffer flow of the integration chip, reducing the concentration of glucose to levels suitable for the glucose biosensor. The buffer used for the flow within the integration chip was chosen to best suit the glucose biosensor best. The concept of the integration chip is shown in Figure 21 and a CFD simulation of diffusion of the glucose in Figure 22.
Fig. 21. Schematic of the measurement methodology behind the biosensor integration chip with a diffusion limiting membrane. A limited amount of analytes diffuses into the fresh buffer through the diffusion limiting membrane and is guided onto a biosensor. The diffusion limiting membrane also prevents contamination of the sample from the internal buffer loop (IV).

Fig. 22. Illustration of a computational fluid dynamics simulation of the diffusion of glucose into the internal buffer flow from sample through the diffusion limiting membrane (created by Ansys CFD).

4.2 Aims and objectives

This chapter aims to develop the necessary technology for the integration of a model glucose biosensor into diverse microbioreactors. Depending on application,
different integration techniques were used in order to perform reliable and representative measurements using the biosensor whilst keeping the bioprocesses within the microbioreactor undisturbed.

For the RTD and lactose hydrolysis monitoring within packed bed microbioreactors a simple integration of the biosensor was used; the utilization of the biosensor was compared to the current state of the art measurement technology.

A biosensor integration chip was developed for integration into a microbioreactor to facilitate the sterilization of the system and addressing the sensitivity and matrix effect issues. An SLA 3D printed microbioreactor was used, however, the integration chip was a transferrable solution.

4.3 Materials and methods

All chemicals were analytical grade and sourced from Sigma Aldrich, Germany, unless otherwise specified.

4.3.1 Residence time distribution monitoring using glucose as a tracer in a packed bed microbioreactor

The packed bed reactors were made of 20 mm PMMA plates with a 0.4 mm thick teflon spacer into which the shape of the reactor was manually cut. The reactors were packed with Novozym® 435 particles (Novozymes, Denmark) and were of four different shapes:

- R1: width 3 mm, length 25 mm, depth 0.4 mm, volume 17 µL
- R2: width 3 mm, length 52 mm, depth 0.4 mm, volume 35 µL
- R3: width 16 mm, length 52 mm, depth 0.4 mm, volume 266 µL
- R4: width 29 mm, length 52 mm, depth 0.4 mm, volume 583 µL

The residence time distribution experiment was conducted in 0.1 M PBS. A syringe pump (Harvard apparatus, USA) was used to generate a constant flow rate of 100 µL/min, followed by an HPLC injection valve with a 10 µL sample loop that was used to inject 50 mM glucose (in PBS) into the flow. Downstream from the injection loop one of the packed bed reactors was installed followed by an integrated electrochemical glucose oxidase biosensor (Figure 23).
4.3.2 **Online monitoring of lactose hydrolysis in a packed bed microbioreactor**

10 beads (approximately 5 mm in diameter) with immobilized lactase (Lentikat’s, Czech Republic) were placed into a packed bed reactor. A syringe pump (Harvard Apparatus, USA) was used to pump the lactose solution in 0.1 M acetate buffer at pH 7 at different concentrations and flow rates. An electrochemical glucose biosensor was integrated downstream from the microbioreactor with a possibility of injecting a diluting buffer to comply with the biosensor’s concentration linear range.

4.3.3 **Cultivation in a microbioreactor with integrated glucose biosensor and optical density biosensor**

The cultivation of *Saccharomyces cerevisiae* (from dried baking yeast, 10 mg/mL) in Milireactor (scheme shown in Figure 24) was done at 30°C in standard YPD (yeast extract, peptone and dextrose) medium spiked with glucose at 20 g/L (1 mL total volume). Optical density was monitored by a calibrated ODsens (as described in Chapter 2.4.6) in a click-on FFF 3D printed form (Figure 27) and glucose using integration chip with a diffusion limiting membrane and an electrochemical glucose biosensor.
oxidase biosensor. A programmable syringe pump (in house made) was used to facilitate the glucose measurements within a PMMA CO₂-laser cut microfluidic chip with an integrated dialysis membrane (8 kDa cutoff).

Fig. 24. Scheme of the Milireactor concept. A – main reaction chamber; B – magnetic stirrers; C – inlet and outlet ports; D – diffusion limiting membrane; E – biosensor integration chip; F – inlet and outlet tubing of the biosensor integration chip; G – integrated electrochemical biosensors; H – magnetic wheel for mixing actuation; I – 3D printed stand.

4.4 Results and discussion

4.4.1 Residence time distribution monitoring using glucose as a tracer in a packed bed microbioreactor

The reactors R1 and R2 had a common width and varied in length whilst R3 and R4 varied in width with a common length. The results of the residence time distribution experiments are shown in Figure 25. For the purpose of negative
control, the inlet and outlet tubings were connected, monitoring the response of the system without an integrated packed bed microbioreactor. The comparison the peaks detected by R1 and R2 to the negative control showed slight changes in the peak shape and distinctive shifts that correspond to the length of the reactor. The shape of the injected glucose plug therefore did not change within the packed bed reactor, suggesting plug flow conditions within both R1 and R2.

The reactors R3 and R4 showed great changes of the shapes of the peaks that were caused by significantly wider geometries in comparison with R1 and R2. There, plug flow was disturbed as the liquid spread throughout the width of the reactors with the apparent dilution effects.

The residence time within the reactor was calculated as the difference in time for the initial increase of the glucose concentration from the baseline for a reactor and negative probe (without reactor). The experimental results were 0.21, 0.35, 2.61 and 4.15 minutes for R1, R2, R3 and R4, respectively. Dividing the packed bed microbioreactor volume with the flow rate of 100 µL/min yields theoretical residence times of 0.17, 0.35, 2.66 and 5.83 minutes for R1, R2, R3 and R4,
respectively, showing a great correspondence ($p = 0.015$). This result can be taken as a validation of the experimental methodology and successful biosensor integration into microbioreactors whilst providing invaluable data for further characterization and understanding of the fluid dynamics phenomena within the packed bed microbioreactors for the collaborating scientists.

Compared to the large scale RTD monitoring reported by Lali et al., where ethanol was used as a tracer measured by an refractive index detector [109], the developed system showed a superior resolution. According to the available data, Lali et al. used a flow cell that had an internal volume of around 50 µL, a volume which is bigger than the total internal volumes of R1 and R2. In comparison, the biosensor integration chamber had an internal volume of 4 µL, assuring an appropriate resolution of the measurements. Additionally, the refractive index of a liquid is dependent on most molecules dissolved in water, not only ethanol. Comparing the calibration curve of ethanol detection by refractive index to the glucose biosensor’s calibration curve, the latter showed significantly higher sensitivity. By using glucose in combination with the glucose biosensor, sensitivity, selectivity and reliability of the measurements were superior.

### 4.4.2 Online monitoring of lactose hydrolysis in a packed bed microbioreactor

The direct glucose measurement methodology was used for monitoring of lactose hydrolysis, yielding glucose and galactose. Based on the balance of the chemical equation, the molar glucose yield equals to the galactose yield as well as the lactose consumption. The experiment was conducted at room temperature using the 0.1 M acetate buffer at pH 7.0. The initial lactose concentration was 5.0 mM and the tested flow rates were 100, 200 and 300 µL/min. The experimental results including the yield calculations are shown in Figure 26.
The electrochemical glucose oxidase biosensor was calibrated prior to the beginning of the experiments in the acetate buffer that was used for bioprocessing and its linearity was extended over 5 mM, making it appropriate for measurements without dilutions.

The results showed the maximum yield just short of 90% at the flow rate of 100 µL/min. With the increase of the flow rates the yields decreased, which could be attributed to shorter residence times within the packed bed reactor leaving insufficient times for complete conversion of lactose. The process would have been quicker if the enzyme had been immobilized on the surface of the beads thus avoiding the time necessary for the lactose and yielding products to diffuse into and out of the beads, respectively.

As the application of the bioprocess and the developed beads was not only the consumption of lactose but the production of high value galactose, the experiments at higher concentrations continued (bioprocessing at high concentrations is desirable as later concentrating or drying out is not cost effective). Contrary to the expectations, the yields fell to around 10%. As a microfluidic mixing and dilution system was used in this case, it was independently investigated showing an uncompromised efficacy. The reason for low yields was found in the occurrence of oligosaccharides [11]; at high concentrations of glucose in galactose the lactase

Fig. 26. Lactose hydrolysis in a packed bed microbioreactor: detected concentrations at the outlet are directly proportional to the yield (N = 3).
tends to catalyze the production of different oligosaccharides that go undetected by the glucose biosensor.

The behavior of the integrated glucose oxidase biosensor was found favorable for the bioprocess monitoring because of the possibility of rapid, online measurements that required next to no manual labor as the experiments could be automated. Additionally, an HPLC method was developed to validate the results gathered by the biosensor, however the glucose and galactose chromatographic peaks were found difficult to separate and the sensitivity of the biosensor was found to be superior (glucose concentrations below 5 mM were left undetected by the HPLC).

4.4.3 Cultivation in a microbioreactor with integrated glucose biosensor and optical density biosensor

By using the biosensor integration chip, the majority of the challenges – sterilization, stability and matrix effects – were addressed, however additional attention was required regarding the sensitivity. The concentration of glucose in the
buffer flow within the biosensor integration chip had to be kept within the linear range of the integrated biosensor and could be manipulated by the choice of the diffusion limiting membrane and the flow patterns. The latter was found to be simple and controllable with a programmable syringe pump. Three measurement methodologies were developed and characterized: steady state, peak and passive measurements.

**Steady state** measurements require a steady flow rate passing into the biosensor integration chip underneath the diffusion limiting membrane and downstream over the biosensor. During the passage underneath the diffusion membrane glucose diffuses from the cultivation broth the chip is exposed to into the buffer, where the concentration is dependent on the buffer flow rate. At 100 µL/min, linear range of the system was found to be up to 60 mM with the plateau at over 100 mM (Figure 28). As this protocol is steady state, Fick’s first law applies:

\[
J = -D \frac{dc}{dx}
\]

where \( J \) is the diffusion flux, \( D \) is diffusivity, \( c \) glucose concentration and \( x \) the diffusion distance. Diffusion flux is the amount of glucose (in mols) that passes through the membrane of a certain surface area (\( A \)) per time unit (\( \Phi_n \), diffusion flow rate) and can be denoted as \( J = \Phi_n / A \). The diffusion flow rate equals the amount of glucose exiting the chip, hence \( \Phi_n = \Phi_v \times c_B \), where \( \Phi_v \) is the buffer volumetric flow rate and \( c_B \) the glucose concentration of exiting buffer. The derivative of concentration over distance (\( dc/dx \)) represents the difference of the inner glucose concentration (\( c_M \), as in measured fluid) and the one in exiting buffer (\( c_B \)) over the thickness of dialysis membrane (\( x \)). Hence:

\[
\Phi_v \times c_B = -A D \frac{c_M - c_B}{x}
\]

As the buffer volumetric flow rate is preset, the membrane surface area is defined by geometry, the membrane thickness is known, the inner glucose concentration is controlled during the calibration due to the usage of standard solutions and the glucose concentration in the buffer is measured. The only variable left unknown is the diffusivity. Using the experimental data from Figure 28, the diffusion coefficient of glucose through the dialysis membrane was calculated to be \( 2 \times 10^{-15} \) m²s⁻¹.
Steady state measurements can provide high density of data on glucose concentrations within the fluid the chip is exposed to, however the sensitivity is poor at around 50 nA at the maximum signal compared to the standard readout signals that can reach over 1000 nA. Additionally, a continuous pumping of buffer may cause flushing of glucose and other molecules that can diffuse through the diffusion limiting membrane. Within 24 hours of exposure and measurement of a 20 g/L glucose solution, approximately 23 mg of glucose would be lost due to diffusion into the buffer for measurements.

**Peak** measurements were introduced in order to enhance the sensitivity of the system and reduce the amount of diffusion losses at the cost of compromised data frequency. The protocol is based on stopping the buffer flow within the biosensor integration chip for a certain amount of time (hereon referred to as diffusion time) to allow the diffusion to locally increase the glucose concentrations in the buffer volume (plug) stopped underneath the diffusion limiting membrane. As the diffusion time passes, the buffer flow rate is turned back on again, pushing the plug from underneath the membrane over the glucose biosensor that detects a distinctive peak in glucose (as shown in Figure 31). The height of this peak is, based on the Fick’s second law, dependent on the chip geometry (constant), the diffusion time and ultimately on the analyte concentration on the other side of the membrane.
Hence the sensitivity of the system can hence be fine-tuned during the measurements by programming different diffusion times into the buffer syringe pump. The calibration curves for 1, 2 and 5-minute diffusion times are shown in Figure 29.

![Calibration curve](image)

**Fig. 29. Calibration curve of the glucose biosensor in the integration chip with a diffusion limiting membrane in peak mode for 1 (triangle), 2 (square) and 5 minute (circle) diffusion times (N = 3) (IV).**

The frequency of measurements using the peak protocol can be manually set by adding a pause after the measurement. As it is impossible to prevent the diffusion into the buffer during the pause time, the chip must be flushed before the start of the diffusion time. The flushing is seen as another peak by the glucose sensor, which is disregarded (or it can be taken as an extra measurement point if it is within the range of the biosensor). In this way, the measurements of high precision, sensitivity (with signals easily reaching towards 1000 nA) and significantly cut diffusion losses (each 5-minute diffusion time measurement of 20 g/L glucose causes around 0.05 ng loss) can be acquired with the frequency that is chosen by the user.

**Passive** measurements occur as a result of diffusion of glucose or other analyte from underneath the membrane to and over the integrated biosensor while the flow
is stopped. Despite the small cross-section of the connective channel (approximately 0.1 mm²) that slows down the diffusion, the small distance (approximately 10 mm) allows detectable amounts of the analyte over the biosensor. It is seen as a gradual, linear increase in readout from the time when the flow rate is stopped. The gradient occurs because the diffusion processes strive to equilibrate the glucose concentration within the biosensor integration chip. The gradient of this increase was found to be linearly proportional to the concentration of the analyte in the measured fluid (calibration curve is shown in Figure 30).

![Fig. 30. Calibration curve of the glucose biosensor in the integration chip with a diffusion limiting membrane in the passive detection mode (N = 3) (IV).](image)

The passive measurement protocol can only work for a limited amount of time as the biosensor would eventually get saturated, an equilibrium would be reached or the measured concentration would change. It is therefore only suitable in combination with another protocol, usually the peak measurement protocol.

Combinations of all three protocols can provide high density of measurements at minimum diffusive losses. For example, if a peak measurement with a 5-minute diffusion time is taken every 15 minutes, an additional peak measurement can be taken after the pause time, two additional passive measurements can be derived from both pause and diffusion flows while the last source of data points could be a steady flow readout when the pump is on and the readout is stable (Figure 31).
Fig. 31. Diffusion chip sensorgram; glucose sensor readouts in black, buffer flow rate in blue (primary vertical axis) and the diffusive losses in orange (secondary vertical axis). From 0-600 s the system was exposed to 20 mM, 600-1200 s to 50 mM and from 1200 onwards to 100 mM glucose concentration (IV).

After the development of the biosensor integration chip and its measurement protocols, it was tested in the Milireactor, monitoring the glucose consumption. As the optical density sensor detected a slow growth of *Saccharomyces cerevisiae*, the glucose biosensor showed the consumption of glucose (Figure 32).
Compared to the current state of the art, an integrated glucose biosensor was able to provide data on glucose consumption within a microbioreactor, which was previously impossible due to the large sample volumes required for testing when the standard analytical equipment was used. Oliveira et al. reported cultivation of yeast in a diffusion based device, where the local glucose concentrations were assumed to comply with the color intensity when a food dye was diffusing instead of glucose [87]. Milireactor’s integrated system for glucose monitoring provides more accurate, reliable and real time data from a calibrated biosensor in an noninvasive manner for the housed bioprocess. The system is transferrable; diverse microorganisms and non-adherent cell lines can be cultivated and more biosensors to monitor other parameters and analytes (e.g. lactate, pyruvate or galactose) can be installed into the biosensor integration chip.

4.5 Conclusions

The glucose biosensor was investigated as a model biosensor integrated in three diverse applications, first to trace glucose for residence time distribution monitoring in a packed bed microbioreactor and finally in a 3D printed device for microbial cultivation. Within the first two applications, the monitoring was
undertaken directly in the bioprocess fluid with an optional dilution system whilst a complex biosensor integration system was developed for the integration into a cultivation microbioreactor. The developed systems and technologies were transferrable to other applications and biosensors for biopharmaceutical process monitoring.

All studies were compared to the reported state of the art and the integration of glucose biosensor, regardless of the application, was found to provide more reliable readouts with the capability of real time monitoring whilst relieving the manual labor and the need for expensive analytical equipment. Low fabrication costs facilitate parallelization and high throughput options, invaluable for research and production in the field of biopharmaceutical processing.
5 Biosensing of folic acid using a molecularly imprinted polymer as a recognition component

5.1 Introduction

Generally, development of biosensors is striving towards novel targets whilst pursuing selectivity, sensitivity and stability. Biological molecules like enzymes, antibodies and aptamers deliver on selectivity and sensitivity but lack in stability both in terms of troublesome sterilization as well as degradation of sensitivity with time, exposure to elevated temperatures, pH, ionic strength or solvents. Additionally, research, development and production of appropriate antibodies and aptamers for biosensors tends to be expensive and labor intensive.

Molecularly imprinted polymers (MIPs) represent a prospective technology of economic development and fabrication of artificial receptors, also known as “plastic antibodies” [112]. Molecular imprinting is a procedure of polymerization of monomers and crosslinkers around a target molecule, leaving behind a cavity of appropriate shapes and exposed complementary functional groups for selective bonding after the template is washed out [71,70]. MIPs can therefore be engineered to have favorable binding capabilities to fit the selectivity and sensitivity criteria for biological recognition components of biosensors, moreover, they exhibit impressive stability characteristics of a polymer material.

Folic acid (FA) was chosen as a target molecule for the development of biosensing technology using MIPs. FA belongs to the vitamin B group (B12) that is essential for humans; a sufficient amount of FA during pregnancy, for example, is known to decrease the occurrence of neural tube defects [113].

A standard of practice for the detection of FA remains chromatography (HPLC and GC). Some attempts at utilization of various forms of MIPs have been reported [114], however few have been transformed into a biosensor or biosensing device.

5.2 Aims and objectives

The aim of the research presented in this chapter is to develop a biosensing device based on MIPs for detection of FA. The objectives involve all steps of development of the anti-FA MIP (choice of solvents, screening of monomers and crosslinkers as
well as optimization of the ratio between the components) and full characterization of the developed anti-MIP (V).

Most MIPs are used for solid phase extraction (SPE) as a sample pretreatment before the detection with chromatographic techniques (e.g. HPLC or GC). A microfluidic SPE device with optical detection, autonomous temperature and flow control was developed for the purposes of the MIP SPE characteristics research with further biosensing prospective.

5.3 Materials and methods

5.3.1 Development of anti-FA MIP

Methacrylic acid (MAA), 4-vinylpyridine (4VPy), vinylbenzyl trimethylammonium chloride (VBTMAC), ethylene glycol dimethacrylate (EGDMA), divinyl benzene (DVB), folic acid ≥97 % (FA), ascorbic acid, 2-mercaptoethanol 99 %, azobisisobutyronitrile (AIBN), silica gel, potassium dihydrogen phosphate and dibasic potassium phosphate were bought from Sigma-Aldrich (Spain). 1-Methyl-2-pyrrolidone (NMP) from Alfa Aesar (Germany), acetonitrile (ACN) from Lab-Scan (Ireland), hydrochloric acid (HCl) and methanol (MeOH) from Panreac (Spain) and dimethyl sulphoxide (DMSO) and technical acetone were acquired from VWR (Spain). Only the solvents that yielded successful results are listed.

4VPy, EGDMA and DVB were purified in silica gel columns in order to remove the polymerization inhibitors, whilst the other components were used as received. The phosphate buffer was prepared from potassium dihydrogen phosphate and dibasic potassium phosphate in Milli-Q water at the concentration of 0.1 M and pH 7.0.

MIPs and non-imprinted polymers (NIP) were synthesized in glass vials in appropriate solvent mixtures to successfully dissolve all the components. First, the appropriately dissolved template (FA, 10 mg) was mixed with an appropriate monomer and incubated at room temperature for 10 minutes for the pre-polymerization process. Then, a crosslinker was added (specific monomers and crosslinkers are listed in Table 3), followed by the addition of 50 mg AIBN. Vials were sealed with septums and left to cool down on ice before 2-minute purging with nitrogen. Polymerization was carried out at 50°C for 24 h. The NIPs were produced following the same procedure excluding the FA.
After 24 h of incubation, the vials were opened and placed into a vacuum oven at 50°C overnight in order to remove the remaining solvents. After that they were smashed and the synthesized polymer was taken out and crushed in a lab mortar. The fine particles that would cause a high pressure drop in SPE cartridges were eliminated by sedimentation in acetone (1 min sedimentation in 50 mm of liquid depth) – the sediment was collected and the supernatant discarded. The polymers were then cleaned using 1 M HCl:NMP (1:1) solution in a lab microwave for 20 minutes at 100°C. Finally, the polymers were further washed with acetone and dried in a vacuum oven at RT overnight (V).

### 5.3.2 Testing and characterization of anti-FA MIPs

The binding properties of the MIPs and the NIPs were established by using adsorption isotherms. These show the relationship between the equilibrium concentration of bound and free target over a certain concentration range and can be generated from equilibrium batch rebinding studies. 10 mg of an MIP or an NIP was added to the 2.5 mL 40 μM FA solution in the 0.1 M phosphate buffer in a glass vial. The vial was sealed and then shaken in an orbital shaking platform for 24 h at room temperature. Then, the FA concentration of the supernatant was spectrophotometrically measured at 280 nm. The best MIP was investigated afterwards at an increased MIP amount (30 mg) and FA concentrations (0.05, 0.1, 0.3, 0.5, 1, 2, 3 and 5 mM) in triplicates (V).

### 5.3.3 Microfluidic MIP-SPE system

The MIP-SPE system (Figure 33) consisted of the following major components: a programmable syringe pump (homemade), an HPLC-style 6-port injection valve with a 100 μL sample loop, an MIP-SPE Peltier thermo-controlled column and a UV optical detector.
Fig. 33. The complete microfluidic MIP-SPE system: a photo with a superimposed structure scheme.

The MIP-SPE column was milled into a block of aluminum that was chosen for its thermal conduciveness and chemical inertness. The internal volume of 12.5 µL was filled with the anti-FA MIP and the particles were contained by a PE 25 µm frit on each side, bolted in place using the O-rings. The column was placed into an FFF 3D printed housing, firmly in touch with a Peltier element, the other side of which was in a ventilated area for heating or cooling, depending on the operation (Figure 34). A water-ice calibrated thermistor was placed into the MIP-SPE column. The system was regulated by a custom developed Arduino® based microcontroller; the MIP-SPE column had a temperature range from -20 to 70°C and the set temperature was contained within 0.05°C margin.
The UV detector was based on a photodiode for the readouts and a UV LED as a light source at 365 nm and bandwidth of 10 nm. The readouts were performed using the same principle as for ODsens (Chapter 2.4.6) but in an FFF 3D printed housing for a flow cell in order to enable continuous measurements in flow downstream from the MIP-SPE column.

The core of the flow cell was fabricated from two 5 mm thick black CO₂-laser cut PMMA plates bonded by a double sided medical grade adhesive (Adhesive Research, Ireland). The core formed a 10.3 mm long channel for light absorption with an internal volume of 7.9 µL. The channel was closed by a glass slide on each side for its transparency within UV light range (Figure 35). The flow cell was
inserted into the UV optical detector device in a way to allow the passage of light from the UV LED to the readout photodiode. During the development, the electronics of the optical detector have been slightly modified in order to optimize the sensitivity of the readouts within the UV light range (the 1 MΩ voltage splitter was replaced by a 30 MΩ one).

Before testing of the anti-FA MIP in the MIP-SPE system, the optical detector was calibrated in the absence of the column (bypass) by injecting the standard solutions of the FA in PBS buffer (156, 312, 500, 625, 1250 and 2500 µM concentrations). The MIPs tested in the microfluidic MIP-SPE system were first packed into the column and installed into the system. The purpose of the system is to find the binding capacity of the tested MIP and this was done by repeated injections of the FA standard until the packed MIP no longer retained the target, ideally seen as an increase of signal from the optical detector.

Fig. 35. Flow cell for continuous optical detection. Top: scheme with the flow path marked in blue and the light path with violet arrows; bottom: photograph of the assembled flow cell.
5.4 Results and discussion

5.4.1 Development of anti-FA MIP

Due to the very high molecular mass of the FA (441.4 g/mol) compared to the utilized monomers (86.1, 105.1 and 211.7 g/mol for MAA, 4VPy and VBTMAC, respectively) and crosslinkers (198.2 and 130.2 g/mol for EGDMA and DVB) the synthesis was only possible with 1:25:250 mass ratio as to avoid unreasonably high masses of the FA (V). More than 40 different solvents and solvent combinations were tested in order to support the polymerization recipes. Empirically, the FA was only soluble in sufficient amounts in DMSO and NMP for further use. Toluene and ACN were found to be miscible porogens with DMSO and NMP and were considered in combinations for dissolving the functional components for the polymerization. While a combination of NMP and ACN worked for most monomers and crosslinkers, VBTMAC required DMSO instead of NMP. The final recipes are listed in Table 3.

The performance of a MIP (or a NIP) was assessed through an adsorption isotherm test, finding a respective binding capacity ($B$):

$$B = \frac{n_0 - n_1}{m}$$

where $m$ is mass of the polymer (MIP or NIP), $n_0$ and $n_1$ initial and final molar quantities of FA in the supernatant, respectively. The molar quantities are determined from the fluid volume $V$, the initial FA concentration $C$ and the final (measured) concentration $C_{fin}$:

$$B = \frac{(C - C_{fin})V}{m}$$

The imprinting factor (IF) was calculated as a ratio of MIP and NIP binding capacities, $B_{MIP}$ and $B_{NIP}$, respectively:

$$IF = \frac{B_{MIP}}{B_{NIP}}$$

An IF higher than 1 signifies a successful molecular imprinting as the binding capacity of the MIP is than higher than the one of the NIP.
First, MIPs and NIPs following recipes 1-6 (Table 3) were synthesized and characterized based on the adsorption isotherm test at the initial concentration of FA at 40 µM. The recipe with the best IF (nr. 5, Table 3) was then tested at different ratios of monomer and crosslinker – recipes 7-10. The complete results of the isotherm tests are shown in Figure 36 (V).

Table 3. Anti-FA MIP recipes and their imprinting factors (V)

<table>
<thead>
<tr>
<th>Polymer nr</th>
<th>Monomer</th>
<th>Crosslinker</th>
<th>Solvent</th>
<th>Imprinting factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MAA (250 mg)</td>
<td>EGDMA (2500 mg)</td>
<td>1:1 NMP:ACN (3 mL)</td>
<td>1.82</td>
</tr>
<tr>
<td>2</td>
<td>MAA (250 mg)</td>
<td>DVB (2500 mg)</td>
<td>1:1 NMP:ACN (3 mL)</td>
<td>0.82</td>
</tr>
<tr>
<td>3</td>
<td>4VPy (250 mg)</td>
<td>EGDMA (2500 mg)</td>
<td>1:1 NMP:ACN (3 mL)</td>
<td>0.71</td>
</tr>
<tr>
<td>4</td>
<td>4VPy (250 mg)</td>
<td>DVB (2500 mg)</td>
<td>1:1 NMP:ACN (3 mL)</td>
<td>1.03</td>
</tr>
<tr>
<td>5</td>
<td>VBTMAC (250 mg)</td>
<td>EGDMA (2500 mg)</td>
<td>5:3 DMSO:ACN (4 mL)</td>
<td>3.98</td>
</tr>
<tr>
<td>6</td>
<td>VBTMAC (250 mg)</td>
<td>DVB (2500 mg)</td>
<td>5:3 DMSO:ACN (4 mL)</td>
<td>2.57</td>
</tr>
<tr>
<td>7</td>
<td>VBTMAC (312.5 mg)</td>
<td>EGDMA (2500 mg)</td>
<td>5:3 DMSO:ACN (4 mL)</td>
<td>3.62</td>
</tr>
<tr>
<td>8</td>
<td>VBTMAC (375 mg)</td>
<td>EGDMA (2500 mg)</td>
<td>5:3 DMSO:ACN (4 mL)</td>
<td>1.11</td>
</tr>
<tr>
<td>9</td>
<td>VBTMAC (250 mg)</td>
<td>EGDMA (3125 mg)</td>
<td>5:3 DMSO:ACN (4 mL)</td>
<td>0.89</td>
</tr>
<tr>
<td>10</td>
<td>VBTMAC (250 mg)</td>
<td>EGDMA (3750 mg)</td>
<td>5:3 DMSO:ACN (4 mL)</td>
<td>1.01</td>
</tr>
</tbody>
</table>

The best combination of monomer and crosslinker was found to be VBTMAC and EGDMA, respectively, with an imprinting factor of 3.98 found at the initial FA concentration of 40 µM. The following test of optimization at different monomer and crosslinker ratios yielded no success, proving that the initial ratio was optimal. Full characterization of the newly developed optimal recipe for the anti-FA MIP followed.

Initially, the adsorption isotherm was extended to the FA concentrations of up to 5 mM (Figure 38) that yielded additional values of IF at each concentration (Figure 39). Impressive characteristics were found with the imprinting factors soaring up to 37 and as the MIP was capable of adsorbing more than 2.5 mmol of FA per gram of the MIP at the initial concentration of 5 mM; in other words, during the adsorption isotherm tests the developed MIP adsorbed a mass of FA higher than its own mass (110 %) (V).
Fig. 36. Complete results of the adsorption isotherm tests. Binding capacity \((B)\) for each MIP/NIP is shown. As the combination of VBTMAC and EGDMA was found to be optimal, further tests of monomer to crosslinker ratio were tested \((N = 3)\) \((V)\).

Next, a kinetic binding study was performed for the MIP and the NIP of the best recipe (VBTMAC-EGDMA). 10 mg of MIP or NIP was separately incubated in a 40 \(\mu\)M FA solution in the 0.1 M phosphate buffer with the FA concentration in the supernatant continuously monitored. From this information, \(B\) at each time point for both the MIP and the NIP was calculated (the results are shown in Figure 37). The MIP was found to reach the binding equilibrium after 20 minutes, making this MIP appropriate for testing in the SPE conditions \((V)\).
Fig. 37. Kinetic binding study of the developed anti-FA MIP at the initial FA concentration of 40 µM (N = 3). Binding capacity (B) plotted over time (V).

Fig. 38. Extended adsorption isotherm test for the developed anti-FA MIP (N = 3). Binding capacity (B) plotted over the initial concentration (C) (V).
5.4.2 Testing of the anti-FA MIP in the microfluidic MIP-SPE system

The developed anti-FA MIP (3 mg) was packed into the microfluidic MIP-SPE column. Despite the clear optical absorption peak of FA at 280 nm, as seen from the spectrophotometric data in Figure 40, 365 nm wavelength UV light was used for the continuous detection of FA as the sensitivity of the photo detector decreases below 300 nm. What is more, low wavelength lights can be absorbed by standard glass that was used for completion of the flow cell. The calibration of the system was done with the column bypassed; different standards of FA in 0.1 PBS have been injected into the system with the injection volume kept at 100 mM (sample loop specification) and the responses of the optical detector were compared and are shown in Figure 41.

Next, binding capability of the anti-FA MIP was tested at 30°C by repeating injections of 1 mM FA in 0.1 M PBS. Due to the presence of the MIP, the initial injections showed only the baseline readouts as all of the FA was adsorbed onto the MIP. The number of injections until a detectable rise in signal was counted and the result corresponded to the adsorption capability of the tested anti-FA MIP of 1.5 mmol/g or 66% of its own mass. At the same initial FA concentration standard adsorption isotherm tests conducted with the dispersed MIP, in comparison, yield lower adsorptions of up to 0.9 mmol/g. The column SPE conditions are not directly
comparable to the standard tests, however, the improvement in the adsorption using the microfluidic MIP-SPE system is clear.

Fig. 40. Absorbance spectrum of folic acid at 125, 62.5, 31.25 and 15.6 µM concentrations seen in the decreasing order of intensity.

Fig. 41. Readouts of the microfluidic MIP-SPE system. Left: calibration using 2500, 1250, 625, 500, 312 and 156 µM FA concentrations, top to bottom; right: initial readout without the MIP (black line, highest peak) decreases with the introduction of the MIP to the baseline, a small peak (dotted line) shows saturation of the MIP.
5.5 Conclusions

The developed anti-FA MIP showed exceptional adsorption capabilities with a likely unprecedented imprinting factor of 37. What is more, in certain conditions the mass of adsorbed molecule could overcome the mass of the MIP itself, but the poor solubility and nonspecific binding also likely played an important role in this case.

The developed microfluidic MIP-SPE system proved as an invaluable tool for the investigation of the MIP behavior in the SPE conditions. Furthermore, the flow cell’s online readout capabilities and optimization of the injection volumes and the MIP masses would allow the transformation of the system into a biosensing one. In this case the MIP-SPE column acted as a bioreactor, miniaturizing the experiments, allowing the parallelization as well as enhancing the adsorption effects. The developed technology is also transferrable to biopharmaceutical downstream processing.

Compared to the current state of the art, the described technology further establishes the value of MIPs as the recognition components for biosensing. Adding that microfluidic technology creates a complete technology for biosensing based on the solid phase extraction principle.
6 Conclusions and future perspectives

Biopharmaceutical industry contributes roughly to half of global pharmaceutical expenditures [6] and is in return changing the prospects of patients suffering from severe diseases [4]. Miniaturization of research – utilization of microfluidics and microbioreactors – reduced the turnaround times and costs as a result of reduced hardware and material requirements [9].

The bottleneck of biopharmaceutical micro-scale processing is the analytics. Integration of sensors to detect and monitor properties like temperature, pH and dissolved oxygen has been thoroughly researched and reported [14], however determination of biochemical composition of micro-bioprocess liquids relies heavily on the standard analytical equipment, thus elevating the costs and time for the analysis and reducing the multiplexing capabilities. As biosensors can provide specific quantitative analytical information their integration into microbioreactors could provide the necessary analytics.

Herein, glucose, lactate, pyruvate and galactose biosensors have been developed and tested. Their functionality was characterized uncovering several challenges to be surmounted before integration into microfluidics: an appropriate stability for long term online monitoring, challenging sterilization due to the presence of sensitive biological recognition components, matrix effects that affect the readouts and too high sensitivity for quantitative detection in high concentrations usually present in the bioprocess fluids.

In order to surmount the challenges of integration of biosensors into microbioreactors, the micro-scale unit operations, like active as well as passive mixing and pumping were investigated including their corresponding fabrication and actuation techniques. Together, the fabrication techniques and the unit operations formed a toolkit that served as a technological basis for overcoming the integration challenges.

The electrochemical glucose sensor was used as a model biosensor to monitor the concentrations of this crucial microbiological nutrient. A solution that surmounted all challenges, a microfluidic biosensor integration chip, involved the contact of the tested fluid on one side (e.g. fermentation broth in a cultivation microbioreactor) and the internal buffer flow on the other over a diffusion limiting membrane. The latter allowed a small amount of analytes to pass through the membrane into the buffer flow, reducing the concentration to meet the linear range of an integrated biosensor. The diffusion limiting membrane also allowed a non-sterile integration of the biosensor after previous sterilization of the system; the
internal buffer flow was thus non-sterile with the diffusion limiting membrane preventing the contamination of the rest of the system. Three developed detection protocols accounted for the versatility and transferability of the system to house different processes and biosensors as the sensitivity and possible loss of analytes through the integration chip could be fine-tuned. The functionality of the system was then tested on a 3D printed custom made microbioreactor featuring mixing, temperature control and optical density measurements for cultivation of *Saccharomyces cerevisiae* in YPD medium; the glucose readouts proved to be stable and reliable, and what is more, a rapid and automatic online detection of glucose allowed previously unfeasible process control in a microbioreactor.

Lastly, the technology of molecular imprinting of polymers was investigated to produce artificial recognition components that would allow sterilization and further adaptation for biopharmaceutical process monitoring. An anti-folic acid MIP was developed and characterized before the investigation of solid phase extraction-based detection device.

All reported developments and technologies represent pioneering attempts of integration of biosensors into microbioreactors for biopharmaceutical processes. In the future, integrated biosensors could replace or reduce the use of standard analytical equipment, relieving the analytical bottleneck and allow further parallelization of operations and processes. Furthermore, the online detection capabilities of biosensors allow real time monitoring, providing rich bioprocess data and enabling the micro-process control.
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