Anna Sophie Schwarz, BSc

Study of hemoglobin as oxygen vector in reactions catalyzed by immobilized enzymes

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Univ.-Prof. Dipl.-Ing. Dr. techn. Bernd Nidetzky
Dr. Juan M. Bolivar-Bolivar

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Sauerstoff abhängige Enzyme werden in der Biokatalyse zunehmend relevanter. Die Versorgung von Enzymen mit molekularem Sauerstoff mit hoher Durchsatzrate ist problematisch aufgrund niedriger Sauerstoff-Löslichkeit in wässrigen Lösungen. Dieses Problem vergrößert sich jedoch noch, wenn auf mesoporösen Trägermaterial immobilisierte Enzyme verwendet werden. Einige Lösungsansätze beschäftigen sich mit dem Reaktor- oder Katalysatordesign. Ein relativ neuer Ansatz ist die Verbesserung der Substratlöslichkeit durch den Einsatz eines Sauerstoffvektors. Sauerstoffvektoren sind meistens organische Lösungsmittel, welche leicht mit Sauerstoff angereichert werden können. Hämoglobin, ein gut charakterisiertes Sauerstoff-Transport Protein, ist eine vielversprechende Alternative solcher organischen Lösungsmittel. Das Ziel dieser Arbeit war es eine Strategie für den Einsatz von Hämoglobin in Sauerstoff verbrauchenden Reaktionen zu entwickeln und seine Eigenschaften Sauerstoff freizusetzen zu untersuchen. Die Freisetzung von Sauerstoff durch Hämoglobin und seine Peroxidase-Aktivität konnte bewiesen werden. Die Immobilisierung von Hemoglobin und Glucose Oxidase (GOX) als Cross-linked enzyme aggregates (CLEAs) und auf mesoporösen Trägermaterial Relizyme™ sowie ihre Effektivität wurde untersucht. Der Sauerstoffabnahme in der flüssigen Phase und im Trägermaterial konnte durch den Einsatz von Hämoglobin und Katalase reduziert werden. Die Gluconsäure-Konzentration konnte unter Sauerstoff limitierten Bedingungen verdreifacht werden, verglichen mit Reaktionen ohne Sauerstoffvektor.

Abstract

Oxygen dependent enzymes are relevant in biocatalysis. The supply of enzymes with molecular oxygen at high transfer rates is problematic due to poor oxygen solubility in aqueous phases. This causes low oxygen concentration in bulk and low oxygen transport from gas into liquid phase. The problem is even more pressing if enzymes immobilized on mesoporous are used, since there is an additional transport step from liquid into solid phase. There are some approaches to address these problems, based on reactor or catalyst design. A relatively new approach is the enhancement of substrate solubility by implementation of an oxygen vector. Oxygen vectors are compounds implemented as second phase in reaction, usually inert organic solvents like fluorocarbon, which are easily saturated with oxygen and can enhance oxygen transfer into aqueous phase. A promising alternative as oxygen vector is hemoglobin, a well-characterized oxygen transport protein. The aim of this thesis was to develop a strategy for the implementation of hemoglobin in reaction with oxygen consuming glucose oxidase (GOX), and to characterize its oxygen release properties. The oxygen release of hemoglobin and its peroxidase activity could be proved. The immobilization of hemoglobin and glucose oxidase as cross-linked enzyme aggregates or on mesoporous Relizyme™ was performed and its effectiveness characterized. The oxygen depletion in bulk and in carrier was lowered by the application of hemoglobin in combination with catalase. The gluconic acid production rate could be increased three-fold under oxygen limited conditions, in comparison with reactions without oxygen vector.

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*"**The single greatest cause of happiness is gratitude.**"*

Oscar Auliq Ice

Contents

1 Introduction

1.1 Problem of oxygen limitation in aqueous phase

Oxygen is a key substrate in aerobic processes, either in microbial cultures or oxygen consuming biocatalytic reactions. A significant number of enzymes, namely oxidases, monooxygenases and dioxygenases, require oxygen as electron acceptor. These reactions are industrially relevant but diverse problems complicate their implementation. The oxygen supply during biocatalytic reaction for example, is one of the most important reaction engineering challenges due to low oxygen solubility and oxygen transfer rate (OTR) from gas into aqueous liquid phase at ambient conditions [1]. This leads to significant lower reaction rate and less productivity, making bioprocesses inefficient. The OTR occurs from gas to liquid and from liquid to solid particle like cells or carrier for solid support of enzymes. To solve these problem, reaction engineering can be applied to optimize reaction conditions for increased yield and productivity [2].

1.2 Oxygen transfer rate between gas-liquid phase

The transfer of oxygen from gas phase to liquid solution is described by the Whitman two-film theory [3] which assumes that both, the gas and liquid phase contains a stagnant layer at their interface. Inside this stagnant layer oxygen transport is defined by diffusion along a concentration gradient, which is the driving force. The mass-transfer through stagnant gas layer is fast and negligible compared to the diffusion through liquid stagnant layer. However, poor oxygen solubility in bulk allows only low concentrations, which leads to low oxygen diffusion [4]. Apart from the difference between oxygen concentrations, the OTR further depends on mass-transfer coefficient through liquid film and interfacial area for oxygen transition. Mass-transfer coefficient and interfacial area can be combined into one empirical constant, the volumetric mass transfer coefficient k_La.

The k_La is a function of energy dissipated to the media, gas hold up and media rheology. It correlates with power input per volume (P/V), the liquid velocity (μ), the superficial gas velocity (v_s) and oxygen gradient (equ.1), [1].

$$OTR = k_La\,(P/V, v_s, \mu) * (C^{sat}_{O2} - C_{O2})$$

Equation 1

C^{sat}_{O2} oxygen concentration in liquid at saturated state

C_{O2} oxygen concentration at gas-liquid phase boundary

$(C^{sat}_{O2} - C_{O2})$ oxygen gradient - driving force for mass transfer

The mass-transfer coefficient k_La is used to characterize the oxygen transfer capability of reactors. At a small k_La, the ability of the reactor to deliver oxygen to the enzyme is limited [5]. The oxygen concentration of aqueous solutions like potassium phosphate buffer PPB, at a temperature of 30 °C is about 240 µM. This low oxygen concentration is clearly substrate limiting for the fast working GOX (100,000-250,000 U/g), which indicates that oxygen is the reaction bottleneck [6].

1.3 Oxygen transfer rate between liquid –solid phase

Heterogeneous biocatalysis is often used in bioprocessing utilizing immobilized enzymes or cells. Apart from biofilms, macroscopic flocs, cross-linking and others, immobilization on solid support or gel entrapment are typical approaches, which can be used if cells or enzymes do not spontaneously flocculate or attach to solid surfaces. The immobilization allows the use of enzymes in continuous operation and has beneficial influence on stability and down-stream processing. Immobilized catalysts can be easily recycled, are more resistant to mechanical agitation, pH or hydrolysis. Reaction can be stopped instantly without the need for heat inactivation or the addition of other stopping reagent by removing the immobilizate from reaction mixture. Immobilization on solid support can be divided into the following categories: Coupling of enzymes, which contains immobilization on carrier or cross linking and entrapment, which can be done in matrix or by membranes. Despite other types of carriers, the immobilization on mesoporous carrier is one of the most efficient one [7].

Mesoporous carrier like Relizyme™ are widely used in industry and research. They offer a high surface area for high enzyme loading, protection of mechanically agitation and simplification in continuous application of enzymes and down-stream processing. They can be homogeneously distributed in bulk phases and have a certain pore size range and distribution. But their use can also lead to drawbacks like activity loss during immobilization, due to incomplete attachment, affected stability and intrinsic activity. Further problems are low substrate diffusion and mass-transport limitations into the carrier, leading to low activity and space-time yield [8], [9]. The mass-transport limitation into carrier is one of the main problems of porous carrier. Within an

ideally mixed reactor a stagnant layer forms around each carrier particle. The liquid in this layer and the liquid inside of particles are therefore not mixed like liquid within bulk and thus molecules can only pass this layer by diffusion. If the substrate is fully converted by the immobilized enzyme, a substrate gradient from bulk into particle forms, having its lowest concentration in the middle of the particle resulting in a substrate limitation. This internal mass-transfer limitation leads to low apparent activity. The oxygen gradient itself is a driving force for substrate diffusion into particle. This means, that for substrate that is poorly soluble in reaction volume, a high gradient is difficult to achieve and the mass-transport limitation into porous particle intensifies. The improvement of biocatalysts that suffers from this conditions is therefore a critical quest in bioprocess technology [10].

CLEAs

Another approach of immobilization is the cross-linking process, which links lysine residues of enzymes by adding cross-linking agent, generating a highly porous enzyme-agent scaffold that could be compared to a sponge. The cross-linking of enzymes is a method derived from CLECs, the cross-linking of enzyme crystals. The precipitation that prepares enzyme for cross-linking is a simple and less expensive method that could also purify enzyme from crude cell extracts. The production of CLEAs can therefore combine purification and immobilization [11]. The CLEA appearance is influenced by preparation conditions. The size and density of CLEAs depends the concentration of the precipitation stock. This has an impact on the substrate transfer into the CLEAs [12]. Further parameters are the molar ratio of cross-linker to enzyme and pH-value, which affect the activity, stability and particle size. The cross-linking efficiency depends on each enzyme´s unique surface structure and therefore on number of lysine residues. For enzymes with few lysine residues at its surface or stocks with low enzyme concentrations, additive proteins are offered to increase lysine residues offered for cross-linking. A common non-enzymatic additive for cross-linking is bovine serum albumin. This should prevent leaking of cross-linked proteins into aqueous media.

1.4 Strategies to increase the oxygen transfer rate

Tools in bioprocess technology to improve OTR are reactor and aeration design, catalyst design and enhancement of substrate solubility.

1.4.1 Conventional methods to increase OTR and k_La by reactor aeration design

Strategies to improve OTR in bulk by reactor design

There are different tools in bioprocess technology to improve the OTR in reactions that are mostly implemented in aerated stirred tanks. Factors that are affecting the oxygen transfer in reactors are bubbles and their diameter/surface and therefore the interface as well as sparing, stirring and media properties. These include bubble formation, gas dispersion, bubble coalescence, viscosity, stirrer speed and gas flow rate. Other factors are antifoam agent, temperature, gas pressure and oxygen partial pressure and the presence of macromolecules.

The characteristic of bubbles in aeration can influence k_L by increasing the interfacial area a. One of the most important properties of air bubbles in fermenters is their size. By dispersing gas into many small bubbles and increasing the surface, oxygen transfer can be significantly improved. Smaller bubbles have a slower bubble-rise velocity and remain longer in liquid, increasing the time for possible oxygen transfer. Furthermore, the gas hold-up is increased, which is defined as the fraction of working volume of the reactor occupied by entrained gas. Besides these advantages, there is a practical limit of bubble size and they are also not fit for use in viscous non-Newtonian broths, where small bubbles remain lodged. Bubbles with 2 to 3 mm diameter and less suffer from more rigid sphere behavior without internal gas circulation and therefore lower k_L. There are also differences in how bubbles are sparged and dispersed in a reactors and how bubbles coalescent.

The increase of OTR by physical processes like higher power input, increased aeration and faster stirring is correlated with a high mechanical agitation. Unfortunately, even stable enzymes are prone to damage by mechanical and chemical degradation. They have a natural reaction window of required conditions of temperature, aqueous environment, pH and other factors. This makes the improvement of OTR in combination with biocatalysts very difficult. Actions to improve the OTR could lead to lower activity or enzyme degradation [13].

1.4.2 Strategies to improve the oxygen transfer rate by solid support design

There are different approaches to improve biocatalysts that suffer from low activity and effectiveness. One example is the development of solid support fitting for each biocatalyst which is a difficult task. Samples can only be taken from bulk and reaction inside of particles cannot be observed or measured. The implementation of an immobilized enzyme as heterogeneous biocatalyst strongly benefits from the availability of suitable process analytical technologies. A new approach is sensing solid support material that change detectable signal due to pH value or O_2 concentration in particle. This allows the comparison of different solid support types to develop the best biocatalyst design like pore shape and size.

Another general problem of immobilization is the undirected link between enzyme and solid support. Immobilization occurs between carrier and certain surface groups found on enzymes. Activity loss occurs if the active sides of enzymes get blocked by carrier or if flexibility gets decreased after immobilization. This applies especially for carriers that offer a surface unsuited for the attachment of enzymes and reporter molecules. The directed immobilization of enzymes by noncovalent tag like Zbasic2 allows an efficient operation in highly selective manner on silica for example. The Zbasic2 is a strongly positively charged three-helix bundle of ~ 7kDa size that is used to immobilize different enzymes including oxidase [14], [9].

Traditional porous carrier are usually macroporous with a pore diameter >50nm, which would allow the enzyme to enter. The current approach however is the use of mesoporous supports with pore diameters ranging between 2 and 50 nm. The smaller pore range is thought to be enzyme stabilizing while not restricting in performance. When characterized, studies showed that pore diameter less than 10 nm lead to a general decrease in protein loading. On the other hand, pore diameters greater than 100 nm lead to decreased protein loading due to reduction of the available surface area. The pore size of a catalyst ideally matches with enzyme size and structure [13].

1.4.3 Increase of substrate solubility

Higher solubility of oxygen and therefore increased $k_{L,}$ can be achieved by change in temperature, pressure and presence of other solutes in solution.

Oxygen is better soluble at low temperatures, which are barely feasible for most bioprocess conditions, due to low apparent enzyme activities. Therefore, the application of immobilization is very limited. The implementation of higher pressure in bioprocess have shown promising results by expanding the process window, achieving dissolved oxygen concentrations up to 43 mM. Enhanced pressure during bioprocesses can be achieved by improvement of technology, namely implementation of pressurized flow reactor [6]. The use of this reactor requires though elaborated bioprocess technology and has to be adapted thoroughly to biocatalysts to be used.

Another approach was the enrichment of aqueous phase with oxygen by peroxide degradation, which proved to be difficult due to its enzyme inactivating properties [15].

The presence of other macromolecules on the other hand, which can adsorb at gas-liquid interphase and cause a reduction of mass-transfer into liquid phase, could also cause an enhancement of interphase [16].

1.4.4 Use of oxygen vectors

A relatively new approach to enhance oxygen solubility is to increase the OTR, by the use of an oxygen vectors, namely by implementation of a second, immiscible liquid phase that does not interfere with the biocatalyst.

The increase of interphase between oxygen rich and oxygen poor phase leads to an improved k_La. This is done by mechanically emulsification of solvent to create small bubbles with a maximized surface area. The solvent takes up oxygen from gas phase and releases it at the interface between gas and liquid phase. Oxygen vectors have already been successfully implemented for oxygen supply in cell cultures. They are mostly organic solvents like perfluorocarbon, which possesses an oxygen solubility of about 3.7 mg/l or 118 mM [17], [18], [19]. This set up has been tested on microbial cultures and showed a high benefit for the oxygen supply.

Like before, the drawback of this approach, is the necessity of high mechanical agitation for emulsification of solvent. They also require a lot of attention in design and development of improved and efficient large-scale aerobic fermentation processes. Immiscible organic solvents

are not feasible as oxygen vector for bioprocesses since the high agitation leads to a swift enzyme degradation. The size of oxygen vector droplets, occurring from emulsification, makes them unable to penetrate porous carrier and therefore not suitable for heterogeneous bioprocesses.

1.4.5 Hemoglobin as oxygen vector

1.4.5.1 Concept of hemoglobin as an oxygen vector

While oxygen vectors can provide the bulk with additional oxygen, the main problem, the limitation into the carrier, would still remain, since bubbles of oxygen vector can´t intrude the carrier. OTR into particles can only enhanced by a higher oxygen gradient from the bulk to carrier. The idea of this project was to use hemoglobin, an oxygen carrying protein found in higher organisms. As a protein it can be immobilized on carriers and it does not only increase the surface between oxygen rich and poor phase, but is soluble in aqueous phase and can directly release oxygen.

Human hemoglobin has a size of 65 kDa and is soluble in aqueous solution in concentration up to 100 mg/ml and higher, making it an excellent oxygen vector. The aim of this work was to implement hemoglobin and determine its suitability as an oxygen vector in the glucose oxidase reaction.

1.4.5.2 General information about hemoglobin and its properties

Hemoglobin is an iron-containing oxygen transport metallo-protein in red blood cells of most vertebrates. It containes four heme groups, each able to take up and release one molecule of O_2. It´s affinity to oxygen depends on various factors like partial pressure (gas pressure in liquid), pH-value, 2,3-bisphosphoglycerate concentration, temperature and the concentration of unusual hemoglobin species like methemoglobin (oxidized hemoglobin), carboxyhemoglobin or fetal hemoglobin [20]. Partial pressure and 2,3-bisphosphoglycerate have the highest influences on oxygen affinity. 2,3-bisphosphoglyerate reduces the affinity to oxygen to enable fetal hemoglobin to take up oxygen from adult hemoglobin.

At high partial pressure, hemoglobin has a strong affinity to oxygen and is highly saturated. At low partial pressure, the affinity to oxygen decreases and hemoglobin starts to release oxygen. At a partial pressure of 100 µM, hemoglobin is approximately 90 % saturated with atmospheric oxygen molecules.

The velocity of oxygen release depends on the saturation state of hemoglobin. If oxygen saturation of hemoglobin is plotted against partial pressure, a sigmoidal curve is shown (fig. 1), [21].

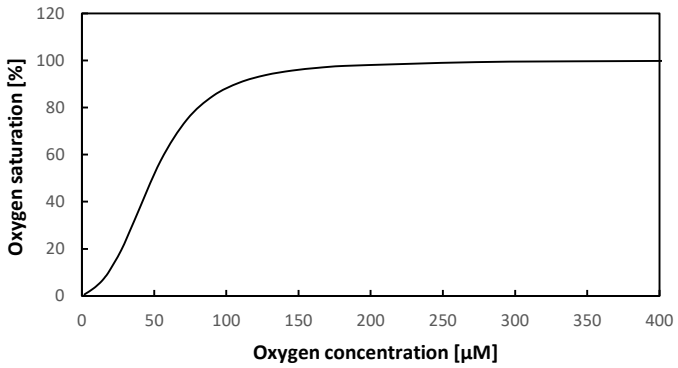

Figure 1*: Oxygen saturation state of hemoglobin as a sigmoidal curve dependent on oxygen concentration in liquid at neutral pH without oxygen affinity changing factors like 2,3-bisphosphoglycerate.*

The use of hemoglobin would likely lead to an increase of the total amount of substrate that would be available for the reaction which ultimately results in a higher product concentration at the end. The higher substrate concentration during reaction, the higher the apparent activity of the enzyme. Hypothetically hemoglobin could be implemented in two ways: as a simple transport protein when dissolved in aqueous phase, that takes up oxygen at high partial pressure at the gas-liquid interface and release oxygen to the unsaturated solution. Or hemoglobin could be immobilized and work as an oxygen transporter into the carrier, stimulated by the oxygen gradient that occurs during the reaction. Hemoglobin would take up oxygen from the oxygen saturated bulk side and releases it on their oxygen lacking side.

1.5 Model reaction for implementation of hemoglobin as oxygen vector

Glucose oxidase (GOX) was used as model enzyme for the implementation of hemoglobin as oxygen vector. It catalyzes the transformation of glucose and oxygen to glucono-lactone and hydrogen peroxide.

Glucono-lactone is unstable and reacts immediately to gluconic acid. The GOX has the advantage of being very specific, stable and well characterized. The reaction with GOX has several commercial applications in food biotechnology and other industrial and pharmaceutical applications like the key function in the glucose assay kit combined with catalase to determine

the blood and urine glucose concentration in diabetes treatments. It also has an antagonistic effect against different food-borne pathogens [22]. Glucose is dissolved in aqueous phase very well to concentrations more than 2 M compared to the co-substrate oxygen.

To determine the influence of hemoglobin on the oxygen depletion oxygen concentration over time in bulk and in particle was compared. For this an oxygen sensor and a non-invasive optical sensing device was used to determine the bulk and intraparticle oxygen concentration. The principle of intraparticle measurement is the use of luminescent dye, e.g the tris (4,7-diphenyl-1,10-phenanthroline) ruthenium(II) dichloride, which is excited at a wavelength of 470 nm and it emits at 550 nm by a sensor. This signal gets quenched by oxygen and increases with oxygen depletion and allows to calculate the oxygen concentration in particle [23]. The precise principle will be later explained in the intraparticle measurement chapter

2 Material and methods

2.1 Materials

Table 1: *List of applied chemicals*

Acetone	Roth
Ammonium sulfate	Roth
Epichlorhydrin	Roth
Ethanol	Roth
di-Hydrogen Potassium Phosphate	Roth
Ethylendiamine EDA	Roth
Phosphoric acid	Roth
Sodium acetate	Roth
Sodium borohydride (NaBH4)	Roth

Table 2: *List of materials*

15 ml screw cap tube	Greiner
50 ml screw cap tube	Greiner
50 ml batch reactor, glass	
1.5 ml Safe lock tube	Eppendorfer
15 ml syringe with 15 µ pores	B/Braun

Table 3: *List of enzymes and proteins*

Glucose oxidase A*. niger*	Sigma Aldrich
Bovine liver catalase	Sigma Aldrich
Bovine serum albumin	Sigma Aldrich
Human hemoglobin	Sigma Aldrich

Table 4: List of used devices

Microcentrifuge 5424 with 24 places rotor®	Eppendorf
Oxygen Microsensor OXR430-HS	PreSens
Cary 50 Bio Uv-vis Visible Spectrophotometer	Varian
Rotavapor Laborota 4000	Heidolph
HPLC Merck	Hitachi
L 7100 quatery pump	LaChrom
L 7400 UV-VIS Detector	LaChrom
L 7360 column heater	LaChrom
Luna® 5 µm NH2 100 °A LG Column 250 + 4.6 mm nr. H17-222478 column	Phenomenex

2.2 Methods

2.2.1 Measurement Methods

2.2.1.1 Determination of glucose oxidase activity

The apparent activity during reaction was determined by measuring the initial oxygen consumption rate in the GOX assay by an oxygen sensor, using the principle of partial pressure measurement in solution. Reagents were preheated to 30 °C and mixed at 300 rpm by a magnetic stirrer. For the reaction 200 µl of 2.5 M glucose were mixed with 3.8 ml of 50 mM PBB buffer (pH= 7.0) to an end concentration of 125 mM of glucose. The reaction was started with 20 µl of enzyme suspension or 100 µl containing enzyme immobilized on solid support. [24]. The volumetric activity of measurement was calculated as the Units substrate converted per ml in reaction defined by enzyme volume used ($volume_{sample}$) in a certain reaction volume ($volume_{tot}$) (equ. 2). The specific activity was calculated as activity immobilized per g carrier (equ. 3).

$$Volumetric\ Activity\ [U/ml] = \frac{volume_{tot}\ [ml] *\ slope\ [U * l^{-1}] * dilution\ factor\ f}{volume_{sample}\ [ml] * 1000\ [ml * l^{-1}]}$$

Equation 2

$$Specific\ activity\ [U/g] = \frac{volumetric\ activity\ [U * ml^{-1}] * 1000\ [mg * g^{-1}]}{carrier\ [mg]}$$

Equation 3

2.2.1.2 Determination of gluconic acid concentration per ion exchange chromatography

The gluconic acid forms the gluconate ion at neutral pH and binds to positively charged amino groups of the stationary phase. Elution is achieved by increasing the ionic strength of the mobile phase, which causes reduction of ionic attraction, or by changing the pH to alter the ionization state of the protein.

The Luna® column was equilibrated with a flow of 0.05 ml per min with ddH_2O. The samples were separated at a flow of 1.5 ml/min and a total elution time of 15 min for each sample measured per UV detector. The results were evaluated with a Chromeleon™ chromatography data system. For the calibration a dilution row of 200, 150, 80, 50, 25, 12.5 and 6.25 mM gluconic acid was prepared. The signal of the calibration was linear until 150 mM of gluconic acid (fig. 2) and a peak

found at 3.5 minutes, within a range of 3.0 - 4.0 minutes. The quality of elution time of gluconic acid changed with continuous use of column from 4.0 min to 4.5 minutes.
The calibration of glucose showed a retention time of 2.5 minutes, in at a retention time of 2.0 – 3.0 minutes.

2.2.1.3 Luminescent intraparticle measurement

Particle preparation

To label mesoporous Relizyme™, a dye solution of 10 ml/g carrier was needed. A solution of ruthenium dye (Tris (4, 7-diphenyl-1, 10-phenantroline) ruthenium(II) dichloride) was prepared with a dye concentration of 0.5 mg/ml in buffer. The dye solution was prepared by slowly adding ethanol (100 %, 1/10 of needed endvolume) to dye powder. This solution was slowly transferred into endvolume of 50 mM PPB. The catalyst was dyed for one hour and washed afterwards until no visible dye was lost anymore. Problems regarding the labelling process of particles, like poor dye saturation, lead to noisy signal. Depending on production batch, the Relizyme™ HFA particles showed better or worse dyeing ability. Particles with yellowish color showed high dye absorption, while white colored particles lost most of the ruthenium dye after washing steps.

Intraparticle measurement

The intraparticle measurement was combined with the oxygen measurement in bulk. The setup of the measurement was the same as for the GOX assay. The signal amplitude of the sensor had to be at least above 1,000 for a sufficient measurement. 25 mg of particles per ml reaction volume were prepared in 4 ml of 50 mM PBB for a sufficient signal to noise ratio during measurement. Emitted signal was measured at oxygen saturated start concentration and defined as 100 %. The reaction was started by adding glucose to a concentration of 125 mM. After some minutes of reaction time, GOX was added in excess to remove oxygen and to measure emitted signal at 0 % of oxygen start concentration. The signal measured at start oxygen concentration was stated as phase 1 and after deoxygenation as phase 2. The higher the signal difference between start and end signal, the less noisy the intraparticle measurement [23], [25].

Calculation of oxygen concentration from signal

Measured signal from full oxygen saturation and after removal of oxygen is assigned to oxygen concentration measured by oxygen sensor. 1/tanphase 1 is calculated from signal measured at oxygen start concentration, defined as phase 1 (equ. 4).

$$1/\tan(phase\ 1) = (phase\ 1 * 3.1416/180)$$

Equation 4

The same is done for oxygen-less liquid: 1/tan(phase 2) from signal measured at oxygen end concentration, defined as phase 2 (equ. 5)

$$1/\tan(phase\ 2) = (phase\ 2 * 3.1416/180)$$

Equation5

The slope was calculated by dividing difference between 1/tan(phase1) and 1/tan(phase2) by difference of oxygen start and end concentration (equ. 6).

$$slope = \frac{1/\tan(phase\ 1) - 1/\tan(phase\ 2)}{oxygen\ 1 - oxygen\ 2}$$

Equation 6

The slope, oxygen start- and end concentration and 1/tan(phase) 1 and 2 was used to calculated odenate 1 (equ. 7) and 2 (equ. 8):

$$Odenate\ 1 = 1/\tan(phase\ 1) - slope * oxygen\ 1$$

Equation 7

$$Odenate\ 2 = 1/tan(phase\ 2) - oxygen\ 2 * slope$$

Equation 8

By applying equ. 9 to values of phase measurement oxygen concentration in particle could be calculated.

$$Oxygen\ concentration\ in\ particle\ [\mu M] = (1/tan(phase * 3.1416/180) - odenate\ 1/slope)$$

Equation 9

2.2.2 Characterization of hemoglobin

2.2.2.1 Photometric spectrum measurement

The spectrum of 2.5 mg/ml of hemoglobin dissolved in 50 mM PBB buffer were photometrically measured before and after implementation in reactions with GOX. The GOX assay was used for the measurement and the reaction started with the addition of 50 µl of a GOX solution with 0.2 mg/ml (GOX assay from material and methods). After 5 min of reaction a sample of 100 µl was taken and dissolved in 900 µl PBB in a photometer cuvette and the spectrum measured again. The spectrum measurement included wavelength in the range of 300-700 nm [20].

2.2.2.2 Determination of oxygen release of hemoglobin in unsaturated buffer

50 mM PBB buffer was deoxygenized by negative pressure and heating (40°C 70 mbar, Rotavapor, see materials) to a final oxygen concentration of approximately 50 µM.

Hemoglobin was added to a concentration of 0.2 mg/ml. Reoxygenation over time was measured by an oxygen sensor. Deoxygenized buffer without hemoglobin was used to determine natural reoxygenation of the buffer.

2.2.2.3 Determination of hemoglobin concentration in solution by BCA assay

The standard BCA protein assay kit from Thermofisher Scientific was used to measure protein concentrations. The protein measurement range is from 20 to 2,000 µg/ml and the samples were diluted to meet this range. For the calibration protein concentrations of 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 mg/ml were measured (fig. 3). The assay was prepared by mixing the working reagents A and B in a ratio of 50:1. The protein samples were diluted in PBB buffer (50 mM, pH= 7.0) and measured in duplicates. 0.1 ml of sample was mixed with 2.0 ml of working reagent and incubated for 30 minutes at 37°C. After that, the samples were incubated for 10 minutes, at room temperature to decrease their temperature and photometrically measured at 562 nm (fig. 3). PBB buffer was used as blank instead of water, like advised in the protocol.

2.2.2.4 Determination of peroxidase activity of hemoglobin per catalase assay and oxygen sensor

Peroxidase activity was determined by measurement of oxygen generated by peroxide degradation. The degradation of one mol of peroxide produces half a mol of oxygen. The measurement of oxygen increase allowed to calculate the peroxidase activity.

3,732 µl of 50 mM PBB buffer (pH=7.0) was preheated to 30 °C and 268 µl of 300 mM peroxide solution was added. The reaction was started with the addition of catalase or hemoglobin after stability of peroxide was evaluated. This was done by observation of oxygen concentration. An eventual autodegradation of peroxide was detected by increase of oxygen concentration. To measure full peroxide degradation, enzymatic and hemoglobin activity must be low enough to prevent the formation of oxygen bubbles, which cannot be detected by oxygen sensor.

2.2.3. Immobilization

2.2.3.1 Cross-linking with glutaraldehyde

Precipitation:

A solution of 3.5 M ammonium sulfate was prepared for the precipitation with distilled water at 50 °C. The solution was left at room temperature over night to settle. Afterwards it was set to a pH of 7.3 [26]. A tenfold enzyme stock solution of desired end concentration was prepared in PBB (100 mM, pH=7.3). All reactions were performed at room temperature with pre-chilled solvents. Enzyme aliquot was always mixed 1:10 with ammonium sulfate precipitate (v/v). BSA or Hemoglobin was added as co-cross-linking additives in a quick addition step to the enzyme stock solution, also in tenfold concentration. 0.1 ml of enzyme aliquot was added to precipitant in one fast step and immediately inverted to mix. After this the precipitation was left at room temperature for 40 min at 20 rpm in a rotator. Afterwards samples were centrifugated at 13,000 rpm for 10 minutes.

Cross-linking:

The cross-linking process was started by adding glutaraldehyde with 25 % (w/w) to a concentration of 1.2 % (v/v) to the samples and rotated at room temperature and 20 rpm for 3 h. After 3 h CLEAs were centrifuged for 10 min at 13,000 rpm and the supernatant was discarded. Uncross-linked enzyme and remaining glutaraldehyde got removed with supernatant. The received CLEAs were suspended in buffer and sodium borohydride ($NaBH_4$) added to a

concentration of 100 mM as a reduction step. This was done in a 15 ml tube to prevent an overflow of the reaction, since $NaBH_4$ is very reactive and causes a lot of highly alkaline foam. The closed lid of the safe lock tubes led to an increase of pressure and explosion. The reduction step was carried out for 40 min and the CLEAs were centrifuged again. After two washing steps, CLEAs were dispersed in 0.1 M sodium phosphate buffer (pH 7.3) and stored at 4°C until further usage. The cross-linking created gelatinous CLEAs particles of different size. The cross-linking of hemoglobin without GOX was done identically.

2.2.3.2 Immobilization of GOX, hemoglobin and catalase on Ep403 S porous carrier

Activation of Ep403 S Relizyme™ with amino epoxy groups

2.3 g of Ep403 S carrier were weighted in and 24 ml of sodium bicarbonate buffer, 2.64 ml of acetone and 7 ml of epochlorhydrin (instead of more toxic 1,4-butanediol diglycidyl ether) added and incubated for 12 h. Afterwards it was washed once with 50% acetone and after that with distilled water, until no more acetone scent remained. Relizyme™ 403S HFA particles were used as carrier when indicated [27], [28].

Immobilization

Particles were equilibrated for 5 minutes in 10 mM PBB buffer. Immobilization is usually done by adding 1 ml of enzyme or protein suspension (stock) to 100 mg of porous carrier. For each washing step, the same volume is added and -activity measured. GOX suspension from *Aspergillus niger* was immobilized for 2 hours at room temperature and incubated over night at 4°C. Afterwards the particles were washed until no more activity was measured in the supernatant for the determination of yield of the immobilization.

The immobilization in a buffer of 10 mM PPB showed a high loss in activity, therefore the protocol was changed and 20 mM PBB used instead. The second step was the immobilization of catalase [29]. 74,000 U per g carrier were immobilized. The activity of each washing step was determined by catalase assay in 50 mM PPB buffer and 20 mM peroxide (30% stabilized peroxide) by oxygen sensor. After this, 100 mg/g of hemoglobin was offered for immobilization.

Hemoglobin stock of 10 mg/ml was added to carrier. The amount of immobilized hemoglobin was determined per BCA Assay. The loss of protein was determined by measuring the protein concentration in supernatant and washing steps by BCA assay to calculate the yield. The

immobilization time for hemoglobin was one hour and carrier was not incubated over night. Each immobilizate was reduced in 100 mM solution of sodium borohydride ($NaBH_4$) for 40 minutes to reduce hemoglobin, obtained from Sigma Aldrich in its Fe^{3+} methemoglobin state, to Fe^{2+} oxyhemoglobin. This was necessary to reduce hemoglobin. Pure GOX immobilizates were treated in the same way [24].

2.2.4 Aerated batch reactors

2.2.4.1 Surface aeration reactor

10 to 100 mg/ml of particles were added to 4 ml of 50 mM PBB buffer in a 15 ml tube at room temperature. Reaction was started by adding glucose to 200 mM concentration. Reactor tube was inverted at 20 rpm.

2.2.4.2 Bubble aeration reactor

An oxygen regulation equipment and the oxygen sensor for manual control were assembled together with a batch reactor (fig. 2). 50 ml of 50 mM PBB (pH=7.0) and 200 mM glucose were used for reactions at room temperature. For mixing a magnetic stirrer with a magnetic stir bar was used at 300 rpm. For aeration, the size of bubbles was minimized by a needle tip, put into a pipette tip in the hose. The oxygen concentration was continuously measured by oxygen sensor.

Figure 2: *Reactor vessel for continuous bubble aeration during continuous oxygen concentration measurement.*

3 Results

3.1 Characterization of hemoglobin

3.1.1 Determination of oxygenation and oxidation state of hemoglobin

The oxygenation and oxidation state of lyophilized human hemoglobin from Sigma Aldrich was determined by its photometric spectrum.

Hemoglobin can have different states of oxidation and oxygenation. It possesses four heme groups that are each able to take up or release one molecule of O_2. If the hemes are at ferrous state Fe^{2+}, hemoglobin is deoxygenized and ready to take up oxygen. The uptake of oxygen oxidizes hemes temporary to Fe^{3+}. The technical description of the hemoglobin purchased from Sigma Aldrich, state that their hemoglobin is mostly oxidized to methemoglobin.

Methemoglobin is the result of the oxidation of ferrous deoxygenized hemoglobin to ferric hemoglobin without being loaded with oxygen, which makes it unable to take up oxygen. Hemoglobin shows for each state a different absorption spectrum that can be easily identified by spectrophotometer.

Hemoglobin saturated with oxygen (oxyhemoglobin) has two peaks at a wavelength of 540 and 575 nm (fig. 3A), deoxyhemoglobin one peak at 560nm (fig. 3B) and methemoglobin has no significant peak (fig. 3C), [20].

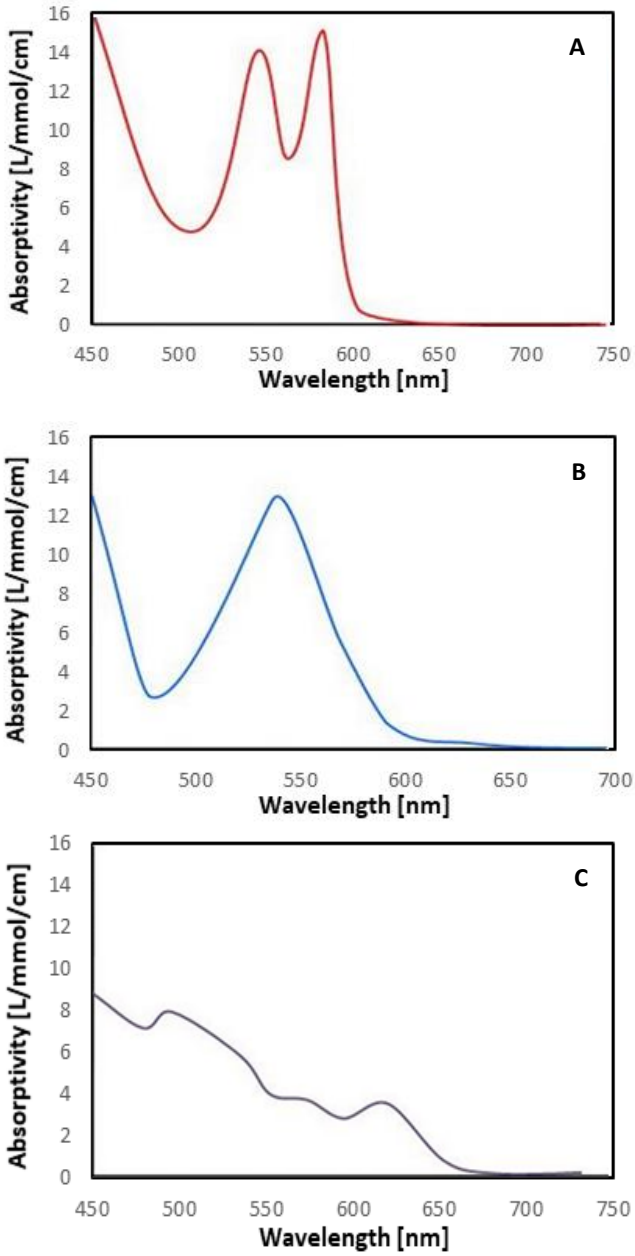

Figure 3*: Absorption spectrum of A.) Oxyhemoglobin with peaks at 540 and 575, B.) Deoxyhemoglobin with a peak at 560 nm and C.) Methemoglobin without significant peak* [20].

To determine the oxygenation state of the sample from Sigma Aldrich the absorption spectrum of hemoglobin was measured. A sample of hemoglobin was implemented in the GOX assay and the spectrum measured before and after reaction with GOX to compare the change of oxygenation state.

Untreated hemoglobin, dissolved to a concentration of 2.5 mg/ml in the GOX assay showed a line without peak. After reaction with GOX, the absorption spectrum showed a slightly flatter line compared of "unreacted" hemoglobin (fig. 4).

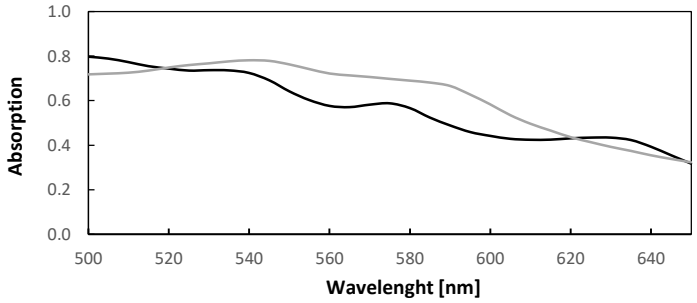

Figure 4*: Photometric spectrum measurement to determine oxidation and oxygenation state of untreated lyophilized human hemoglobin before and after reaction in GOX assay. The black line shows the absorption spectrum of the hemoglobin before the reaction and the grey line hemoglobin after reaction with GOX.*

The flat line indicated that the sample mainly contained of methemoglobin. The state of hemoglobin didn´t change when implemented in reaction, which would be expected of methemoglobin. To be able to take up and release, oxygen, hemoglobin has to be in "active" state. A reduction of hemoglobin should theoretically "re-activate" hemoglobin from oxygenized Fe^{3+} to Fe^{2+} and therefore enable it to take up oxygen from bulk. The previous experiment was repeated with hemoglobin that was reduced in 100 mM $NaBH_4$ for 40 minutes to deoxygenized Fe^{2+} hemoglobin. Since methemoglobin carries no oxygen, hemoglobin is left deoxygenized after reduction. In saturated buffer at concentrations of 240 µM and higher (dependent on temperature) hemoglobin starts to take up oxygen and deoxygenizes the buffer. Hemoglobin was reoxygenized to oxygenized Fe^{3+} hemoglobin by oxygen input into solution by shaking of sample by hand until an oxygen concentration of 240 µmol was achieved. The GOX assay was repeated with the reduced hemoglobin sample.

The spectrum measurement of reduced hemoglobin before reaction with GOX showed two peaks at 540 nm and 575 nm and a flat line after reaction (fig. 5).

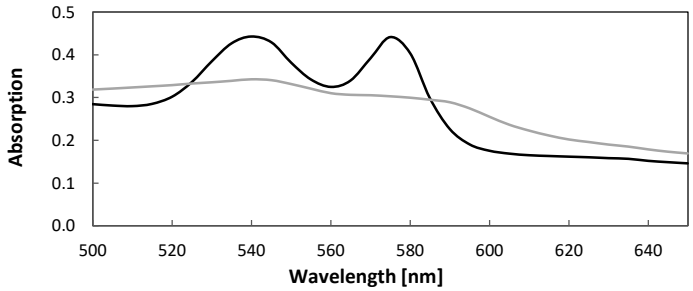

Figure 5: *Photometric spectrum measurement to determine oxidation and oxygenation state of reduced human hemoglobin before and after reaction in GOX assay. The black line shows the absorption spectrum of reduced hemoglobin before the reaction and the grey line reduced hemoglobin after reaction with GOX.*

The two peaks of the hemoglobin indicated the oxygenated state of hemoglobin. After reaction, hemoglobin was transitioned to either methemoglobin or deoxygenated state. The experiment showed that a reduction of hemoglobin is necessary to receive “active” hemoglobin.

The experiment indicated also that at given conditions in buffer, hemoglobin was able to take up oxygen from bulk. At a partial pressure of 100 µM hemoglobin has a high affinity to oxygen and is almost 100 % saturated with oxygen. The partial pressure of oxygen in 50 mM PBB buffer at 30°C is 240 µM, which should cause hemoglobin to take up oxygen from bulk. The oxygenized state of hemoglobin results from oxygen uptake after reduction. The fact that hemoglobin showed oxygenized state at given conditions was a promising start for the purpose of to characterize hemoglobin as an oxygen vector and proved that reduction of methemoglobin by $NaBH_4$ lead to functional hemoglobin. The spectrum measurement however, did not tell how much oxygen was released. For this is was necessary to measure oxygen release by hemoglobin.

3.1.2 Determination of oxygen release of reduced hemoglobin in unsaturated buffer

The hypothesis of hemoglobin as an oxygen carrier depends on its ability of oxygen release at low concentration solution. To study its oxygen release ability, oxygenized hemoglobin was added to unsaturated buffer and the oxygen release at unsaturated conditions measured.

The PBB buffer was deoxygenated by heat and negative pressure (40 °C at 72 mbar) by the rotavapor, to an approximately oxygen concentration of 50 µM. 4 ml of the deoxygenized buffer were transferred into a glass vial, stirred with 300 rpm and the oxygen concentration was

observed by oxygen sensor. Dissolved hemoglobin was added to an end concentration of 0.2 mg/ml and the increase of oxygen measured and compared to the reoxygenation of buffer without hemoglobin.

The oxygen concentration measurement of buffer without hemoglobin increased by 21 µM after 10 minutes while the presence of reduced hemoglobin lead to an oxygen increase of 72 µM in the same time (fig. 6).

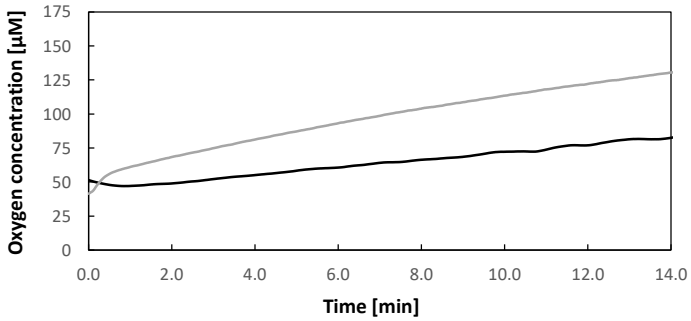

Figure 6*: Measurement of oxygen release of hemoglobin in oxygen unsaturated conditions. Reoxygenation of deoxygenated buffer with and without 0.2 mg of hemoglobin per ml was measured and compared. Black line shows re-oxygenation of buffer without hemoglobin and grey line re-oxygenation with hemoglobin.*

The re-oxygenation of buffer over time was significantly higher with hemoglobin. This proved that hemoglobin releases oxygen in unsaturated buffer and defined it as a suitable candidate for an oxygen vector.

However, the experiment showed an additional effect: 0.2 mg/ml hemoglobin in buffer could carry a maximum 12 µM of oxygen. The difference of more than 50 µmol of oxygen increase between buffer with and without hemoglobin indicated an additional effect than a onetime oxygen release. The last part of hemoglobin characterization was to determine its interference with the product of the GOX assay, the peroxide.

3.1.3 Test on peroxidase activity of hemoglobin

A literature research about hemoglobin found, that hemoglobin has redox-reactive properties and is able to degrade peroxide [30], [31]. A catalase assay with hemoglobin instead of catalase was used to determine its peroxidase activity and how much peroxide is degraded per mg of hemoglobin.

The peroxidase activity of enzymes is usually spectrophotometrically measured by a catalase assay. A solution with peroxide is prepared and the degradation by catalase/peroxidase measured by absorption of developing bubbles. This method was not suitable, since the absorption of hemoglobin in solution was too high. The peroxidase activity of hemoglobin was measured instead by the oxygen sensor. 4 ml of 50 mM PBB buffer were mixed with 20 mM peroxide to an end concentration of 1.6 mM, the concentration usually used for the catalase assay. For each mol of peroxide degraded, half a mol of oxygen is generated (equ.10).

$$1\ Mol H_2O_2\ = \frac{1}{2} Mol\ O_2 + 1\ Mol\ H_2O$$

Equation 10

The change of oxygen concentration therefore allowed to calculate back the amount of degraded peroxide. This method, however, has the disadvantage that oxygen is poorly soluble in aqueous buffer. This means, that at increasing oxygen concentration in bulk, a saturation is achieved that causes the generated oxygen to escape as gas bubbles from bulk without being measured by sensor. The amount of hemoglobin suitable for this measurement, had to be empirical determined.

The oxygen concentration of this peroxide assay was measured for 15 seconds before hemoglobin was added, to determine any auto degradation of peroxide. For the first measurement, hemoglobin was added to an end concentration of 2 mg/ml to reaction and an immediate increase of over 250 µM of oxygen was observed (fig. 7).

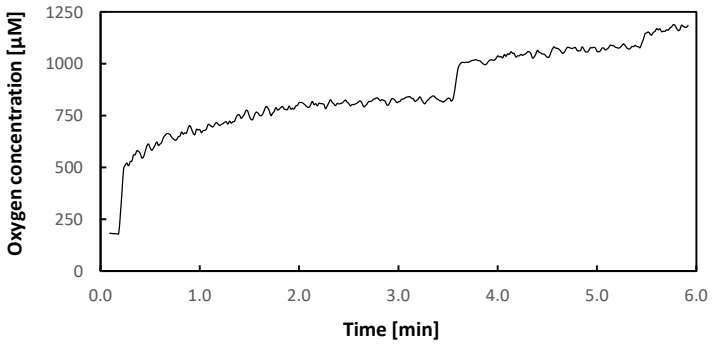

Figure 7: *Peroxidase activity of 2 mg/ml of hemoglobin. Experimental set-up contained of 50 mM PBB (pH=7.0) at 30 °C and 1.6 mM H_2O_2. Hemoglobin was added after 0.25 min.*

A lot of air bubbles were produced and escaped from solution without being measured. After the first massive increase, a continuous rising of oxygen could be observed. A second addition of peroxide caused again a boost of 180 µM oxygen. The excessive production of bubbles prevented an accurate measurement of peroxidase degradation. For the second approach hemoglobin was decreased to a concentration of 0.5 mg/ml.

The peroxide concentration in catalase assay was stable before hemoglobin was added. 30 µM of oxygen were generated by degrading 60 µM of peroxide (fig. 8).

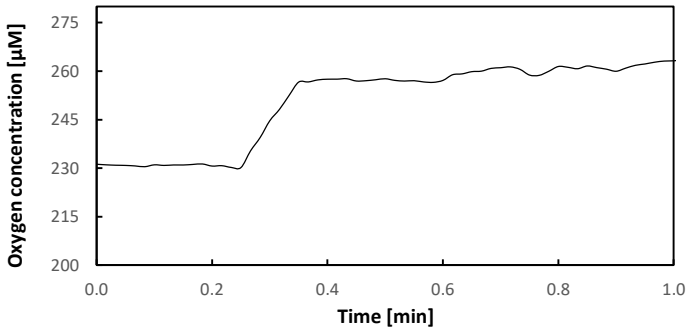

Figure 8: *Peroxidase activity of 0.5 mg of hemoglobin per ml of reaction. Experimental set-up contained 50 mM PBB (pH=7.0) and 20 mM H_2O_2. Peroxide is added and stability observed. Hemoglobin was added after 0.25 min.*

The measurement showed that each active group of hemoglobin degraded more than one molecule of peroxide. Hemoglobin has a molecular weight of 65,000 mg/mmol and possesses 4 heme groups. In a solution with 0.5 mg/ml of hemoglobin, 30.8 µM of active heme groups were available for peroxide degradation (equ.11). The amount of peroxide that was degraded was doubled. The immediate degradation of peroxide by hemoglobin used in reaction with GOX, made the determination of productivity by measurement of peroxidase concentration impossible.

$$\text{Heme groups of hemoglobin } [\mu M] = \frac{0.5\ [mg*ml^{-1}] * 4 * 1000\ [\mu mol*mmol^{-1}] * 1000\ [mg*l^{-1}]}{65{,}000\ [mg*mmol^{-1}]}$$

Equation 11

3.1.4 Conclusions

The photometric spectrum measurement confirmed the oxidized state of hemoglobin and the necessity of reduction. The spectrum showed clearly oxygenized hemoglobin after reduction and reoxygenation. For the following experiments, hemoglobin was always reduced before implemented in reaction.

The results of the oxygen release measurement proved the ability of hemoglobin to repeatedly take up and release oxygen. Being implemented as dissolved protein in a mixed reactor, a molecule of hemoglobin is transported from bulk to gas liquid interphase, like a simple blood circulation system. The high gas pressure at gas-liquid interphase theoretically allows hemoglobin to re-uptake oxygen and release it in bulk. The results proved the oxygen releasing function of hemoglobin in unsaturated bulk.

The velocity of oxygen release of hemoglobin however, depends on partial pressure und therefore on oxygen saturation of buffer. Since oxygen concentration depletes from saturating to oxygen-less concentrations during reactions with GOX, the velocity of oxygen release of hemoglobin also changes. Dependent on velocity of oxygen release by hemoglobin, GOX is provided with an unknown amount. To determine the effect of hemoglobin on activity, depletion in oxygen consuming reactions with and without hemoglobin were compared in the next chapter.

The peroxide degradation by hemoglobin added to peroxidase assay was doubled compared to active heme groups present. Hemoglobin therefore worked as an enzyme and degraded more

than one peroxide molecule per heme group. Since peroxide was added in excess and hemoglobin was added to a certain concentration, oxygen can be produced by peroxide degradation in a higher amount than µM of reductive sides of hemoglobin present in reaction [32].

The characterization of hemoglobin showed promising results for the application as an oxygen vector. The oxygen release and peroxide degradation indicated a kind of enzymatic function that could lead to lower substrate depletion during reaction and prevention of peroxide accumulation. The influence of hemoglobin on reaction by its function had to be determined by comparison of activity and productivity with reactions devoid of hemoglobin. The main question of this thesis is the suitability of hemoglobin as oxygen vector in homogeneous systems. The characterization of immobilization of GOX, hemoglobin and bovine liver catalase was necessary as next step.

3.2 Characterization of immobilization of hemoglobin, GOX and catalase

Two methods of immobilization were characterized, the immobilization on mesoporous carrier Relizyme™ and the cross-linking of enzyme to produce CLEAs (Cross-linked enzyme aggregates). The comparison of different immobilization methods and loadings of GOX was done to study their specific oxygen limitations. Furthermore, the effectiveness of immobilization of hemoglobin and catalase was characterized.

3.2.1 Cross-linking

3.2.1.1 CLEAs-Characterization of cross-linking of Hemoglobin

The cross-linking of hemoglobin stocks of different concentrations was done to characterize the immobilization efficiency.

The immobilization balance of hemoglobin was evaluated by measurement of protein concentration offered for cross-linking and protein that was lost after precipitation and cross-linking step. For this experiment, two stock concentrations with 20 and 10 mg/ml of hemoglobin were precipitated and centrifuged. The supernatant was removed and its protein concentration measured.

The pellet was cross-linked and washed two times, until the last washing step showed an insignificant loss of protein. The first washing step showed a high loss of protein, while during the second washing step almost no hemoglobin was lost. Hemoglobin was immobilized with a yield of 61 and 40 % for the stocks of 20 and 10 mg/ml (table 5).

Table 5: *Characterization of cross-linking process of hemoglobin. BCA assay was used to determine protein concentration for each cross-linking steps of hemoglobin stocks with 20 and 10 mg/ml. Protein concentration of supernatant and washing steps were measured to determine loss of hemoglobin.*

Hemoglobin stocks [mg/ml]	20	10
Supernatant of precipitation [mg/ml]	4.2	3.2
Supernatant of cross-linking [mg/ml]	2.1	2.0
WS1 [mg/ml]	1.4	0.8
WS2 [mg/ml]	0.07	0.02
Cross-linking yield of hemoglobin[%]	61.2	39.8
Cross-linked hemoglobin [mg]	12.2	4.0

The stock with higher hemoglobin concentration also showed higher yield of cross-linked hemoglobin. However, CLEAs made of enzyme stocks with higher concentration has been known as more likely to lose activity. This might be due to higher density, not lower amount of incorporated enzyme, which leads to less mass-transfer into CLEAs during reactions [33]. The problem of high density also might occur to hemoglobin. The influence of CLEA density on activity was characterized by cross-linking of GOX.

3.2.1.2 CLEAs- Characterization of cross-linking of GOX with BSA

The characterization of the cross-linking of GOX was done to study the feasibility. The evaluation was done by an activity balance, which allowed to determine the amount of incorporated enzyme and the efficiency of immobilization. For cross-linking, the enzyme is usually combined with a non-enzymatic protein as additive, which provides lysine residues for linking, to improve the efficiency of cross-linking. BSA (bovine serum albumin) was used as additive for the characterization of cross-linking of GOX.

The necessity to determine the initial reaction rate for activity balance, by measurement of oxygen depletion, was not possible in the case of hemoglobin, since hemoglobin compromises

the activity measurement by oxygen release. The study of activity balance of cross-linking of GOX was therefore done with BSA as co-cross-linking additive.

Samples with two different ratios of BSA to GOX were prepared, to elucidate the influence of additive concentration on the cross-linking process.

Stocks with 0.4 mg/ml of GOX and zero BSA, 2 mg/ml or 4 mg/ml of BSA (ratio of 1:5 and 1:10 to GOX) were prepared and an activity of 85 U/ml measured. For each stock concentration, two precipitation approaches were done. After centrifugation of precipitated protein, activity of supernatant was measured and precipitation yield calculated (equ. 12). The yield showed how much activity was incorporated (equ. 13). The pellet of the second cross-linking approach was re-dissolved in 50 mM PBB buffer (pH=7.0) and re-obtained activity from pellet was measured. The second pellet, which was not re-dissolved, was cross-linked by adding glutaraldehyde. The activity of CLEAs was measured by adding 100 µl of homogeneous mixed CLEAs to 3900 µl of GOX assay with 125 mM glucose to measure activity. The effectiveness of the cross-linking process was determined by calculation of measured activity as percentage of incorporated activity (equ. 14).

$$Yield\ [\%] = \frac{stock\ [U * ml^{-1}] - supernatant\ [U * ml^{-1}\ ]}{stock\ [U * ml^{-1}]} * 100$$

Equation 12

$$Incoporated\ acivity\ [U * ml^{-1}] = offered\ activity\ [U * ml^{-1}]/100 * yield$$

Equation 13

$$Effectiveness\ factor\ \eta = \frac{CLEA\ activity\ [U * ml^{-1}]}{incorporated\ activity\ [U * ml^{-1}]} * 100$$

Equation 14

The activity from dissolved pellet, which had no BSA added, was the highest, while activity precipitation with BSA concentrations decreased. The yield of GOX decreased with higher BSA concentrations as additive from 69.2 % to 49.7 % and 61.7 %. The highest yield had the precipitation without BSA. From activity incorporated in pellet, 15.5 % were re-dissolved if no BSA was co-precipitated while pellets with BSA only showed 12.8 % to 10.2 % of re-dissolved activity.

The activity of CLEAs was elevated from 5.4 % of stock activity to 26.2 and 24.4 % if BSA was co-cross-linked. The effectiveness factor η showed the efficiency of cross-linking dependent on how much enzyme was incorporated and how active this GOX was after cross-linking. η was significantly increased from 7.8 % to 52.8 % and 39.6 % % for CLEAs with BSA (table 6).

Table 6: *Characterization of cross-linking of 0.2 mg/ml GOX with BSA in different ratios by measurement of apparent activity and determination of Yield and effectiveness factor η.*

BSA [mg/ml]	0	2.0	4.0
Stock [U/ml]	83.5	83.5	83.5
Yield [%]	69.2	49.7	61.7
Incorporated activity [U/ml]	57.8	41.5	51.5
Pellet activity [U/ml]	9.0	7.4	5.9
CLEAs [U/ml]	4.5	21.9	20.4
Effectiveness factor η [%]	7.8	52.8	39.6

The amount of activity obtained from re-dissolved pellet indicated higher irreversible precipitation of enzyme in presence of BSA as additive. The amount of precipitated enzyme, defined by yield, was higher for pure GOX CLEAs. η showed that while less enzyme was incorporated in CLEAs, GOX is far more active in CLEAs with co-cross-linking additives. The influence of hemoglobin in reaction with GOX will be determined in a later chapter by comparing oxygen depletion of CLEAs with BSA and CLEAs with hemoglobin as co-cross-linking agent.

3.2.1.3 Conclusions

Hemoglobin CLEAs

The higher yield of cross-linked hemoglobin suspensions of higher concentration was promising, but could have had also the drawback of bigger CLEA particles, occurring from protein clustering. A bigger size of CLEAs also means a denser structure, which could lead to loss of flexibility. [34].

CLEAs made of stocks with higher concentration has been known as more likely to lose activity, due to higher density and low mass transport.

Hemoglobin as well as other enzymes need a certain flexibility to release oxygen. It has two quaternary structures, dependent on oxygenated (R-) or deoxygenated (T-) state [35]. High density in CLEAs could limit the velocity or hinder the oxygen release of hemoglobin-CLEAs.

GOXBSA CLEAs

BSA as additive decreased the yield but increased ŋ. While additives are added to provide more NH_2-residues for a more efficient cross-lining process, yield was unaffected by this. On the contrary, higher stock concentrations resulted in less incorporated enzyme. The characterization however, proved that BSA has a beneficial effect on apparent activity of CLEAs. While yield and effectiveness of GOXBSA CLEAs were satisfying, the cross-linking method had a significant drawback: the manipulation of oxygen depletion made a characterization of GOX and hemoglobin CLEAs impossible. Contrary to porous particles, where enzymes can be immobilized stepwise, stock solutions for CLEAs must contain all proteins that are to be cross-linked. The characterization of GOX and BSA gave the conclusions of a suitable yield and efficiency, but no indications about cross-linking with hemoglobin.

3.2.2 Immobilization on porous carrier Relizyme™

3.2.2.1 Characterization of immobilization of hemoglobin on Relizyme™

The immobilization of hemoglobin on porous carrier was characterized to determine the yield.

100 mg hemoglobin per g carrier was offered and the protein lost during immobilization measured by BCA, from protein concentration of supernatant and washing steps. Since hemoglobin is a non-enzymatic protein, only yield and not activity was determined. The yield was defined as percentage of activity immobilized, calculated by activity that was offered (stock) and activity that was lost during immobilization (supernatant and washing steps) (equ. 15).

$$Yield\ [\%] = \frac{stock\ [mg * ml^{-1}] - sup\ [mg * ml^{-1}] - WS\ [mg * ml^{-1}]}{stock\ [mg * ml^{-1}]} * 100$$

Equation 15

WS washing step

Sup supernatant

A stock with 10 mg/ml hemoglobin in 50 mM PBB (pH=7.0) that showed an intensive dark red color was prepared. 100 mg of porous carrier were incubated with 1 ml of stock for 1 hour. Particles were washed with 20 mM PBB until the washing steps showed no visible color anymore. After that, particles were washed with 50 mM PBB buffer under same conditions.

The first washing step showed the highest loss of hemoglobin with 1.71 mg/ml from the original 10 mg/ml stock. The change from 20 mM to 50 mM of buffer didn´t increase activity loss. 7.36 mg of hemoglobin were immobilized, which comes up to a yield of 73.6 % (table 7).

Table 7: *BCA Assay of the immobilization of 100mg/g of hemoglobin on Relizyme™.*

Stock [mg/ml]	10
Carrier [mg/ml]	100
20 mM PPB	
WS 1 [mg/ml]	1.71
WS 2 [mg/ml]	0.41
WS 3 [mg/ml]	0.14
WS 4 [mg/ml]	0.09
50 mM PPB	
WS 5 [mg/ml]	0.13
WS 6 [mg/ml]	0.07
WS 7 [mg/ml]	0.03
WS 8 [mg/ml]	1.71
Sum [mg/ml]	2.65
Yield [%]	73.6
Hem immobilized	7.36
Hem immobilized per g carrier	73.6

Switching the buffer concentration from 20 to 50 mM PBB buffer induced no further detachment of hemoglobin from carrier, indicating a strong link to Relizyme™.

75 % of hemoglobin was immobilized. The particles showed an intensive dark brown coloring, indicating a saturation of carrier. The amount of 100 mg/g hemoglobin was kept as a standard immobilization concentration for all following experiments.

3.2.2.2 Characterization of immobilization of GOX on Relizyme™

The characterization of immobilization of different GOX loadings on porous carrier was done to determine the efficiency of the immobilization process. The comparison of activity of dissolved enzyme with activity after immobilization can reveal activity loss by immobilization and eventual mass-transport limitations.

Yield and the effectiveness factor η were determined, like for the CLEAs before (equ. 12 and 15). η was determined by apparent activity that was measured, compared to incorporated or theoretically immobilized activity. Yield and η allow to compare the effectiveness of immobilization between different GOX loadings. Activity was measured per oxygen sensor. 2.5 mg/ml of particles were added and the volumetric activity of particles with immobilized GOX measured. The specific activity of U/g was calculated from volumetric activity U/ml by amount of added particles per volume (equ. 2 and 3).

Immobilization of low loadings

The first immobilization was done to study the efficiency of immobilization of low GOX loadings.

Catalyst loadings of 70, 40, 20, 8, 4 and 2 U/g were prepared and the apparent activity of 2.5 mg/ml particles measured by GOX assay.

The yield of this low catalyst loadings decreased from ~ 60 % to ~35 % for increasing loadings. The η for GOX loadings between was well over 100 % for all loadings (fig. 9).

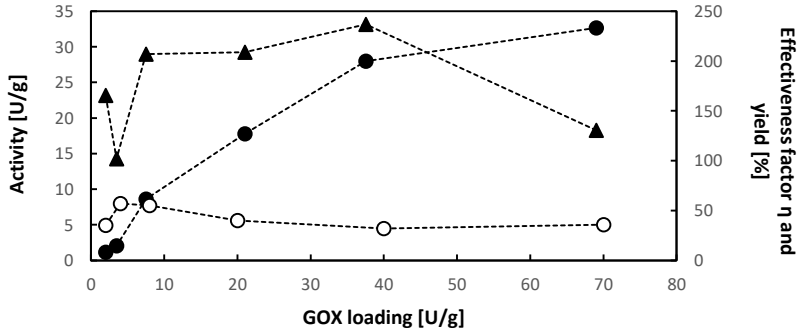

Figure 9*: Activity, effectiveness factor η and yield of immobilized GOX with a loading of 2-70 U/g on Relizyme™. The activity is shown as •, the effectiveness factor η as ▲and the yield as ○.*

The immobilization of GOX of catalyst loadings proved to be efficient. η shows the effectiveness of immobilized enzyme. In the absence of mass transport limitations and other activity compromising factors, η increases to a maximum of 100 %. When compared to apparent activity, the high η values indicated no significant loss of activity of enzyme by immobilization nor mass-transport limitations. The immobilization of loadings between 2 and 70 U/g led to full efficiency and no loss of activity of immobilized enzyme or oxygen limitation in particle. Values of more than 100 % of η are without significance, caused by a lack of proper activity balance. GOX loadings of 70 U/g and less proved to be not substrate limited by diffusion into particle. The next experiment was done with medium high loadings to determine the influence on η and characterize the immobilization.

Immobilization of medium loadings

The first immobilization should show the efficiency of immobilization for medium loadings with GOX. The immobilization of medium loadings should reveal how activity and η are influenced by higher enzyme loss and mass-transfer limitation.

GOX loadings of 350, 150 and 80 U/g were immobilized and immobilization and activity measurement was done as usual.

The immobilizates showed a linear increase of activities of 24, 46 and 149 U/g when plotted against loadings. Yield decreased from almost 100 % to under 80 %. η ascended with increasing loadings from 40 % to almost 60 %, while the activity showed a linear increase (fig. 10).

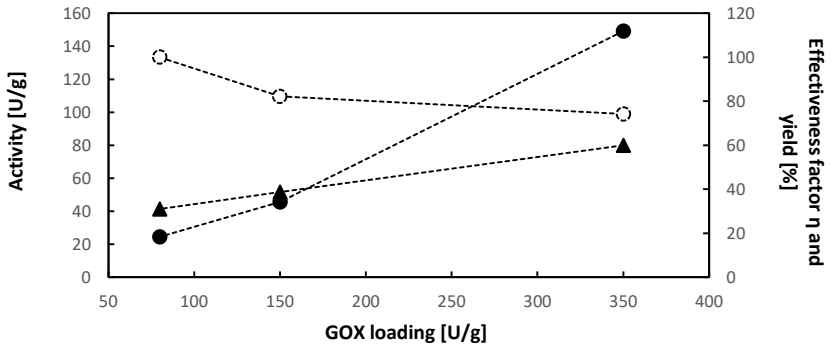

Figure 10*: Activity, effectiveness factor η and yield of immobilized GOX with a loading of 80, 150 and 350 U/g on Relizyme™. The activity is shown as •, the effectiveness factor η as ▲and the yield as ○.*

A possible explanation for the high yield compared to the previous experiment with lower loadings could be that the calculation of the yield was compromised by loosely immobilized enzyme. Enzyme that diffuses after detachment into bulk influences the apparent activity to be higher since mass-transport limitations do not occur for dissolved enzyme. This activity loss of enzymes led to a yield higher calculated than actual yield, which conversely increased η.

At loadings between 80 and 350 U/g of GOX, η was reduced and apparent activity suffered from mass-transport limitations. The results identified the used loadings as feasible for the implementation of hemoglobin in reactions of mild oxygen limitation of immobilized GOX. The increase of loadings in a new immobilization was planned as next experiment to fully characterize of mass-transport and the trend of η.

Immobilization of high loadings

An immobilization of high GOX loadings was done to elucidate carrier saturation and mass-transport limitations of high velocity. GOX loadings of 300, 400, 700, 1100, 1600 and 2400 U/g were offered to carrier.

The percentage of immobilized GOX was between ~ 30 and 56 %, independent of catalyst loading. When apparent activity of immobilized enzyme was compared to theoretically immobilized activity, a high η of ~200 -250 % was calculated for the lower catalyst loadings and a fast decrease for GOX loadings over 500 U/g (fig. 11). The highest loading of 2400 U/g, showed the lowest η of 13 %.

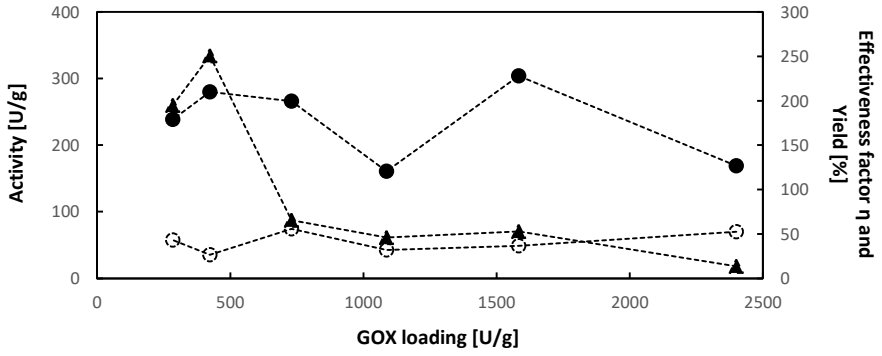

Figure 11*: Activity, effectiveness factor η and yield of immobilized GOX with a loading of 300, 400, 700, 1100, 1600 and 2400 U/g on Relizyme™. The activity is shown as •, the effectiveness factor η as ▲and the yield as ○.*

The activity showed no increase but a plateau when plotted against loadings. High enzyme loadings usually result in low effectiveness due to high reaction rate and low substrate diffusion into porous particles. It could be also caused by either carrier saturation. [36]. The constant yield however, proved that mass-transport limitation caused the stagnant intrinsic activity. The high mass-transport limitations showed the limit of loadings for the immobilization of GOX. The optimal loading was determined as 400 U/g and lower.

3.2.2.3 Conclusions

The yield of different loadings was mostly stable and never fell under 50 %. The efficiency of enzyme incorporation was confirmed. The results showed that the Relizyme™ solid support allows a high range of different GOX loadings without a significant drop of yield.
The comparison of different GOX loadings on Relizyme™ showed a linear increase of apparent activity up to 20 U/g. This slope eases into a plateau for loadings higher than 300 U/g. This activity plateau was the highest specific activity that could be reached without any improvement of bioprocess.

The comparison of η allowed a characterization of mass-transport limitation and their negative influence on apparent activity. For the highest loading of 2500 U/g of GOXwas the immobilization barely effective, with η dropping fast towards zero. GOX loadings under 70 U/g showed no significant mass-transport limitations. For GOX loadings between 80 and 350 U/g mass-transport limitations occurred and showed a negative impact on η but allowed still distinct differences of apparent activity between loadings. This means that apparent activity depended on loading and substrate diffusion into carrier and defined loadings between 80 and 350 U/g as optimum to use in reactions with implemented oxygen vector.

The characterization of immobilization of GOX on Relizyme™ allowed to determine conditions to be used for the work of this thesis; the determination of suitability of hemoglobin as oxygen vector in reactions with substrate limited, immobilized enzyme.

3.3 Effect of hemoglobin on apparent activity

3.3.1 Determination of effect of hemoglobin on oxygen depletion in bulk

The hypothesis of hemoglobin as an oxygen carrier depends on the ability of oxygen release at low partial pressure in solution. It was to be determined how hemoglobin can be best implemented in reaction to show the highest benefit. This was done by comparison of oxygen depletion of bulk of reactions with and without hemoglobin to detect the influence on apparent activity.

The oxygen consumption by GOX should trigger oxygen release by hem and therefore improve oxygen limited reactions. The influence of hemoglobin on depletion by release of oxygen during reaction made a direct activity measurement is impossible. The measurement only shows an apparent activity that can be compared with activity of reactions with GOX.

3.3.1.1 Reactions with dissolved hemoglobin and GOX

Different concentrations of hemoglobin with fixed concentration of GOX

The influence of reduced hemoglobin on oxygen depletion in a reaction with dissolved GOX was to be determined. The goal was to compare how apparent activity was influenced by hemoglobin in different concentrations present in reaction. The optimum concentration of hemoglobin that should be implemented in reaction was determined.

A GOX stock 0.20 mg/ml were prepared and 20 µl added to start the reaction in the GOX assay with 2.5 mg/ml, 7.5 mg/ml, 10.0, 15.0, 20 mg/ml or 25 mg/ml of hemoglobin.

The results showed no significant effect on oxygen depletion, with the highest concentration of hemoglobin causing a decrease of apparent activity from 32.2 to 26.6 U/ml (table 8).

Table 8*: Activity in reaction with 0.2 mg/ml of GOX and different hemoglobin concentrations shows how apparent activity was affected. 20 µl of 0.2 mg/ml dissolved GOX and different concentrations of dissolved hemoglobin were added for reaction.*

Hemoglobin [mg/ml]	0	2.5	7.5	10.0	15.0	20.0	25.0
Activity [U/ml]	32.2	33.1	36.4	33.6	32.0	32.0	26.6

The implementation of hemoglobin in reactions with dissolved GOX showed no noteworthy effect. A possible explanation is that dissolved GOX is only limited by oxygen concentration and activity in reaction. To determine a potential effect of hemoglobin, GOX had to be implemented

in a form, where it is exposed to substrate limitation. Like previous mentioned in the introduction, this occurs to GOX immobilized on porous particles. Mass-transport into this solid support leads to substrate limitation and therefore lower activity of GOX.

Fixed concentration of hemoglobin with different concentrations of GOX

It was to be determined if different velocities of oxygen depletion influences oxygen release by hemoglobin. For this reactions with different GOX concentrations were compared.

GOX stocks with concentrations of 0.05, 0.1, 0.15 and 0.5 mg/ml at 0.2 mg/ml of hemoglobin concentration were prepared and oxygen depletion measured.

The results showed inconsistencies for the oxygen depletion. Activity for reactions with increasing GOX concentration weren´t enhanced. Only two reactions showed lower oxygen depletion of reactions with hemoglobin, the ones with 0.1 and 0.15 mg/ml of GOX. Oxygen depletion by 0.2 mg/ml of GOX increased from 21.0 to 45.8 U/ml (table 9).

Table 9: *Activity of reaction with 2.5 mg/ml of dissolved hemoglobin concentration and different concentrations of dissolved GOX.*

GOX concentration [mg/ml]	0.05	0.10	0.15	0.20
Activity GOX [U/ml]	10.7	26.6	26.3	21.0
Activity of GOX with hemoglobin [U/ml]	11.0	19.8	23.1	45.8

The oxygen depletion in reactions with GOX showed no increase according to enzyme concentration. For reactions with hemoglobin, a lower oxygen depletion was expected, but results were too inconsistent for an effect to be evaluated. Reactions with dissolved enzyme would naturally show less oxygen limitation than for immobilized enzymes. For the next step, GOX was immobilized, to test on effect of hemoglobin on oxygen depletion.

3.3.1.2 Reactions with hemoglobin and immobilized GOX

The effect of hemoglobin on apparent activity and oxygen supply of immobilized GOX was studied.

Two methods of immobilization were already characterized. Both, CLEAs and porous carrier Relizyme™ were used to elucidate the effect of hemoglobin on oxygen depletion. While mass-

transport limitations into porous particles are well known [32], [37], [5], they are less characterized for CLEAs. Both methods were applied to elucidate the potential effect of hemoglobin.

Hemoglobin and GOX cross-linked enzyme aggregates

CLEAs of different GOX concentrations with hemoglobin or BSA as additive in a fixed ratio to enzyme were cross-linked to compare apparent activity.

GOX stocks of 1 mg/ml, 2 mg/ml and 4 mg/ml and additive in a tenfold ratio were prepared.

When apparent activity of different immobilizates were compared, CLEAs with hemoglobin showed a clear decrease. Oxygen depletion of CLEAs with stocks of 1 mg/ml of GOX was the same, regardless if cross-linked with hemoglobin or BSA. Oxygen depletion for high GOX concentration was clearly lower if combined with hemoglobin. For CLEAs with 4 mg/ml stock was oxygen depletion almost divided by half (table 10).

Table 10: *Activity of reaction with CLEAs with different cross-linked GOX concentrations and BSA or hemoglobin in a tenfold ratio to GOX.*

Cross-linked GOX concentration [mg/ml]	1.0	2.0	4.0
Cross-linked additive concentration [mg/ml]	10.0	20.0	40.0
Activity of GOX with BSA [U/ml]	22.2	51.4	144.5
Activity of GOX with hemoglobin [U/ml]	21.5	42.5	80.8

The measurement of volumetric activity proved that oxygen depletion of cross-linked GOX was significant reduced by hemoglobin. This effect increased for higher cross-linked GOX concentrations. Hemoglobin seemed to have a higher benefit under more oxygen limiting conditions. In the next step, these results were to be compared with reactions with GOX immobilized on porous carrier.

Dissolved or immobilized hemoglobin and GOX immobilized on porous carrier

The immobilization of different GOX loadings in combination with hemoglobin was done to determine how oxygen depletion was influenced under different mass-transport limitations.

2.5 mg/ml particles were used for kinetic measurement. Reduced hemoglobin was implemented either immobilized on carrier or dissolved in bulk. 100 mg/g of hemoglobin was offered to carrier with a yield of 80 %. Reaction with hemoglobin contained each 0.2 mg/ml of reaction volume.

Low loadings

Catalyst loadings of 2, 4, 8, 20, 40 and 70 U/g were prepared. Apparent activity influenced by hemoglobin was compared to activity of GOX mono immobilizate, used as the baseline of reaction for the comparison.

Activities of 1.2, 2.0, 8.6, 17.8, 28.0 and 32.6 U/g were measured for the GOX mono immobilizate. When activity of immobilized GOX was plotted against catalyst loadings, it showed a curve that eventually eases into a plateau. The activity of loadings under 20 U/g increased steadily. Activity of GOX baseline was compared with that of immobilized and dissolved hemoglobin.

The results showed a slightly faster increase of oxygen depletion for immobilized GOX and dissolved hem of lower loadings. The implementation of immobilized hemoglobin lead to decrease of oxygen depletion to a third of that of the GOX baseline (fig. 12).

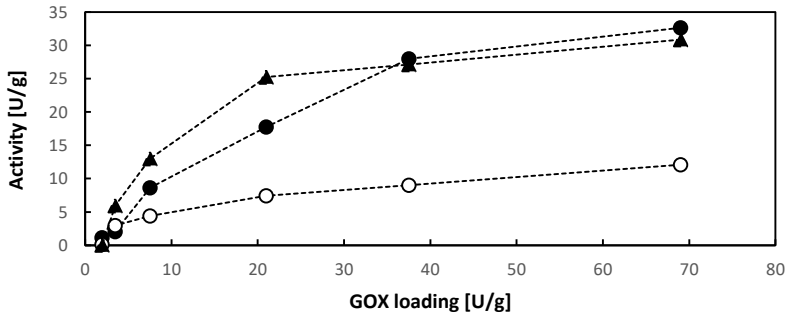

Figure 12*: Comparison of apparent activity of reactions with GOX and hemoglobin, implemented in either immobilized or dissolved form. 2, 4, 8, 20, 40 and 70 U/g GOX were immobilized. Reactions contained 2.0 mg/ml of hemoglobin. Immobilized GOX is shown as •, GOX with immobilized hemoglobin as ○ and GOX with dissolved hemoglobin as ▲.*

Loadings under 20 U/g achieved an activity with almost linear slope and therefore highest efficiency of product vs loading for immobilized GOX. The presence of dissolved hemoglobin seemed to have a different effect than immobilized hemoglobin. While immobilized hemoglobin decreased oxygen depletion as expected, dissolved hemoglobin boosts apparent activity. Hemoglobin in reactions with low catalyst loadings showed a significant influence on the oxygen depletion. An immobilization with higher GOX loadings should reveal further influences on apparent activity.

Medium Loadings

GOX loadings of 350, 150 and 80 U/g were immobilized. Hemoglobin was immobilized and its concentration in reaction as well as the amount of carrier as described in reaction set-up (p.39).

Activities of 31, 39 and 149 U/g were measured as GOX baseline. The comparison of oxygen depletion showed only small differences. The oxygen depletion of catalyst with immobilized GOX and dissolved hemoglobin was again the highest (fig. 13). Activity of all immobilizates was increasing linear.

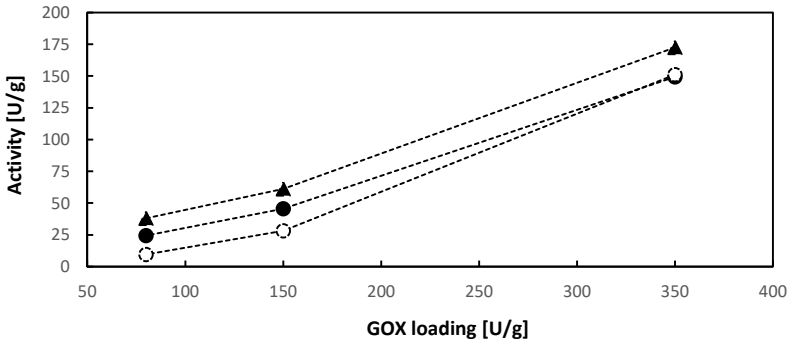

Figure 13*: Comparison of apparent activity of reactions with GOX and hemoglobin, implemented in either immobilized or dissolved form. 80, 150 and 350 U/g GOX were immobilized. Reactions contained 2.0 mg/ml of hemoglobin. Immobilized GOX is shown as •, GOX with immobilized hemoglobin as ○ and GOX with dissolved hemoglobin as ▲.*

The similar activities of different immobilizates could be due to higher oxygen consumption of reaction, compared to oxygen storage of hemoglobin.

The almost linear slope of apparent activity of this experiment indicates that mass-transport limitations could be further increased by higher loadings. For the next experiment, high GOX loadings were immobilized to compare influence of hemoglobin on apparent activity under very high mass-transport limitations.

High Loadings

To determine the effect of hemoglobin on apparent activity, GOX loadings of 300, 400, 700, 1100, 1600 and 2400 U/g carrier were immobilized.

The apparent activity for increasing loadings of GOX were measured as 239, 280, 266, 161, 304 and 169 U/g. Apparent activity of reactions with hemoglobin was very inconsistent but show no distinct difference between reactions with GOX (fig. 14).

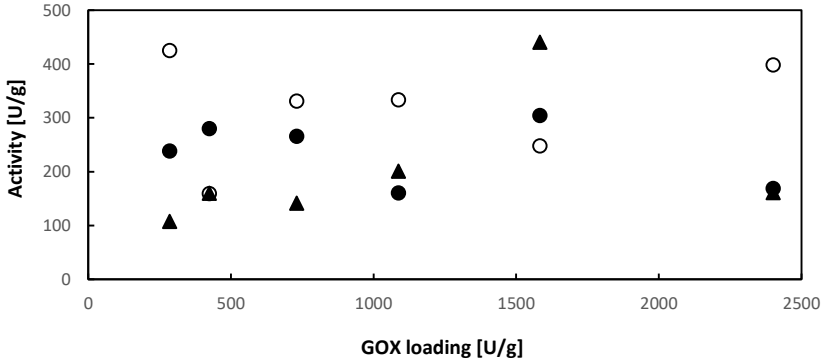

Figure 14: *Comparison of apparent activity of reactions with GOX and hemoglobin, implemented in either immobilized or dissolved form. 300, 400, 700, 1100, 1600 and 2400 U/g GOX were immobilized. Reactions contained 2.0 mg/ml of hemoglobin. Immobilized GOX is shown as •, GOX with immobilized hemoglobin as ○ and GOX with dissolved hemoglobin as ▲.*

Reasons for the plateau of apparent activity could be mass-diffusion problems or enzyme inactivation by high concentrations of peroxide as by product of reaction [15]. The activity improving effect of hemoglobin, that was determined in earlier reactions, was non-existent for loadings higher than 400 U/g. One possibility is that the oxygen supply by given hemoglobin concentration was too limited. Another option was the inactivation of hemoglobin by high peroxide concentrations. Fast activity and mass-transport limitations correlate with high peroxide production and led to accumulation in particle.

The optimum loading for the determination of beneficial effects of hemoglobin was determined between 70 and 400 U/g, introducing mass-transport limitations into reaction but making impacts on apparent activity clearly distinctive.

For the following experiments, loadings between 80 and 350 U/g were used to set-up suitable reaction conditions. In the next step, catalase was implemented to elucidate the effect of hemoglobin on oxygen depletion under optimized conditions of protection against peroxide and oxygen recycling.

3.3.2 Determination of effect of hemoglobin and catalase on oxygen depletion in bulk

The implementation of catalase is an effective method to prevent peroxide accumulation in reactions with GOX. The co-immobilization with oxidases has already been studied and its beneficial effect was characterized sufficiently [24], [38], [39]. The catalase generates half a mol oxygen and water per degraded mol peroxide, is effective and fast. The prevention of peroxide accumulation should lead to a protection of GOX and eventual of hem during reaction and has an additional effect of oxygen recycling.

The use of catalase can be distinguished by addition of dissolved catalase and immobilized catalase. Dissolved catalase can be applied in excess in bulk, but has no proximity to immobilized enzyme and immobilized hemoglobin.

The immobilization of catalase is connected with a loss of enzyme, and activity loss by immobilization, but proximity to GOX and hemoglobin and therefore a likely higher effectiveness for reactions with high mass-transfer limitation that would otherwise lead to peroxide accumulation. The effect of catalase was determined by comparison of oxygen depletion in bulk. The measurement of oxygen concentration of reactions with and without catalase allow to compare the oxygen supply from liquid phase.

3.3.2.1 Reaction with dissolved or immobilized hemoglobin and dissolved catalase

By addition of dissolved catalase, the effect of oxygen recycling in bulk was determined in combination with hemoglobin. The net effect of catalase was determined by implementation in reaction with GOX mono-immobilizate.

For the experiment with dissolved catalase GOX loading of 80, 150 and 350 U/g were immobilized. Catalase was added to a concentration of 2.2 mg/ml.

Activities of 31, 39 and 149 U/g were measured as GOX baseline. Oxygen depletion of particles with 80 U/g loading showed very similar activity for all immobilizates. This changes for higher GOX loadings. Immobilizates that included hemoglobin and or catalase had significant less apparent activity than the GOX baseline for loadings of 350 U/g. The lowest oxygen depletion is achieved for GOX with dissolved hemoglobin and catalase (fig. 15).

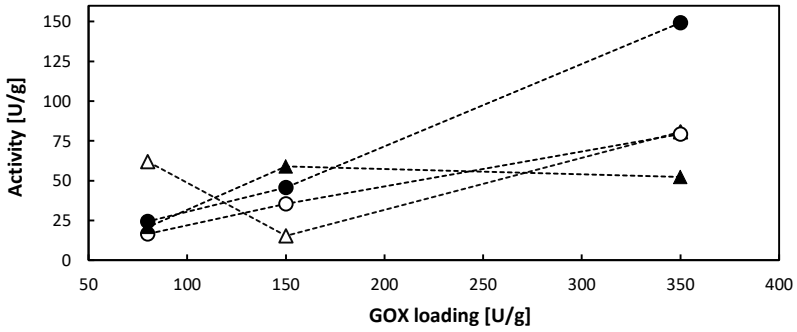

Figure 15: *Comparison of apparent activity of reactions with GOX, hemoglobin and catalase. Loading between 80 and 350 U/g of GOX. Reactions contained 2.0 mg/ml hemoglobin, either dissolved or immobilized and 0.55 mg/ml of catalase. Immobilized GOX is shown as •, GOX with catalase as Δ, GOX with immobilized hemoglobin and catalase as ○ and GOX with dissolved hemoglobin and catalase as ▲.*

Dissolved catalase in bulk was used to recycle oxygen and provide GOX with substrate. GOX with dissolved catalase is therefore expected to show less oxygen depletion. The comparison of the GOX baseline with GOX immobilizate combined with catalase confirmed this. Hemoglobin is also able degrade peroxide, but the effect was negligible compared to the much more effective catalase in excess, if implemented in dissolved form. Any additional influence on oxygen depletion by hemoglobin, would therefore be due to oxygen release or transport by hemoglobin. The results of reaction with dissolved hemoglobin confirmed this.

For GOX with immobilized hemoglobin, peroxide accumulation and oxygen limitation in carrier are probably improved in particle, by direct proximity. Dissolved catalase in bulk would slow down oxygen depletion in bulk, but mass-transport limitations would slow down degradation of peroxide in particles. The implementation of dissolved catalase would only lead to a decreased oxygen depletion in bulk for all immobilizates.

3.3.2.2 Reaction with dissolved or immobilized hemoglobin and immobilized catalase

Catalase was additional immobilized and the influence on oxygen depletion in combination with hemoglobin determined. The net effect of immobilized catalase was determined by comparison of GOX baseline and GOX with catalase.

200 U/g of GOX, 74,000 U/g of catalase and 100 mg/g of hemoglobin were offered to carrier. An apparent activity of 83 U/g for GOX mono-immobilizate was measured. Activity of catalase in washing steps was determined until loss was negligible. A yield of about 72 % of catalase was achieved (table 11).

Table 11*: Immobilization and characterization of approx. 75,000 U/g of catalase on Relizyme™*

Loading [U/g]	74,820
Stock [U/ml]	7,482
Supernatant [U/ml]	961
Washing step 1 [U/ml]	842
Washing step 2 [U/ml]	173
Washing step 3 [U/ml]	109
Immobilized activity [U/ml]	5,397
Yield [%]	72

Oxygen depletion of catalysts that involved catalase and/or hemoglobin was significant lower than the GOX baseline, except for immobilized hemoglobin and catalase, which showed higher apparent activity (91 U/g). The lowest oxygen depletion was measured for catalyst with GOX and catalase (45 U/g) and GOX with immobilized catalase and dissolved hemoglobin (51 U/g) (table 12).

Table 12*: Oxygen depletion of 200 U/g of GOX loading with and without immobilized catalase immobilized or dissolved hemoglobin. Imm.= immobilized and diss.= dissolved.*

GOX [U/g]	83.4
GOX and catalase	45.1
GOX and imm. hemoglobin	67.1
GOX, catalase and imm. hemoglobin	90.9
GOX and diss. hemoglobin	75.5
GOX, catalase and diss. hemoglobin	50.8

Regarding peroxide, dissolved catalase was expected to have less enzyme preserving effect. Due to mass-transport limitations into particle, peroxide accumulates inside of carrier, where it inactivates GOX. Peroxide that that diffuses into bulk is probably a much lesser problem to enzyme, since concentration would be much more diluted.

The effect of oxygen recycling was expected to be very efficient for dissolved catalase, by direct proximity to substrate, leading to reduced oxygen depletion for all immobilizates with dissolved catalase. A faster oxygen depletion for immobilized hemoglobin and catalase was unexpected. Oxygen consumption and recycling were happening inside of particles. The observation of oxygen depletion in bulk was insufficient to determine the effect of hemoglobin, since it gave no indication about oxygen concentration inside of the particles. For this reason, a new method was used, the intraparticle measurement, which allowed measurement of oxygen concentration inside of porous particles.

3.3.3 Effect of hemoglobin and catalase on oxygen depletion in porous carrier

The comparison of oxygen depletion in bulk and particles was essential to show the influence of immobilized hemoglobin and catalase. Oxygen concentration in bulk and in particle were measured simultaneously during reaction for an accurate comparison of oxygen depletion.

The reaction was done in the usual GOX assay set-up. For the intraparticle an optical fiber was used to measure the signal emitted by luminescent dye. The particles were dyed with Tris(4, 7-diphenyl-1, 10-phenantroline) ruthenium (II) dichloride, a luminescent dye. This dye gets excited by light of 470 nm wavelength and emits at 550 nm. The presence of oxygen in liquid quenches this signal, while the depletion of oxygen leads to signal increase.

The link of measured signal with defined phases of maximum and zero oxygen concentration, allowed a continuous kinetic measurement of oxygen depletion (fig 16), [23], [40].

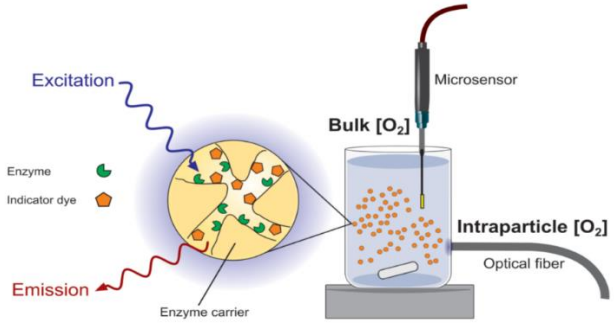

Figure 16: *Concept of intraparticle oxygen measurement of porous particles labelled with luminescent ruthenium dye (Tris (4, 7-diphenyl-1, 10-phenantroline) ruthenium (II) dichloride). Reaction with GOX, immobilized on porous solid support in buffer under continuous stirring with simultaneously measurement of oxygen depletion in bulk by oxygen sensor. Dye is excited by light at 470 nm wavelength and emits at 550 nm. Oxygen concentration in particle is measured by phase shift of signal due to signal quenching by oxygen.*

Immobilizates were prepared, one with GOX and catalase, and one with additional hemoglobin. The influence of hemoglobin on oxygen depletion in bulk, protected by catalase, was to be studied. The oxygen depletion of immobilizate with GOX and catalase was measured as baseline.

Loadings of 30 and 300 U/g of GOX, 7,400 U/g of catalase and 100 mg/mg of hemoglobin were offered to carrier. After immobilization, particles were labelled with luminescent ruthenium dye after immobilization of all proteins. Oxygen concentration in bulk and in carrier was measured simultaneously. 25 mg/ml of particles were added to the GOX assay for a sufficient signal to noise ratio during measurement. Reaction was started by adding glucose and measured for some minutes. After reaching low oxygen concentration, measurement was finished by removing oxygen by adding GOX excess (see intraparticle measurement in material and methods).

The reaction with 300 U/g GOX and catalase showed a steady decrease oxygen of 82 U/g in reaction. The oxygen concentration inside of carrier dropped immediately after reaction start from 240 µM to a concentration of under 50 µM. This concentration was stable until oxygen concentration in bulk was exhausted. A wide gap between oxygen depletion in bulk and in carrier was observed (fig. 17 A).

In the reaction with hemoglobin was the oxygen depletion in bulk reduced to 45 U/g. Oxygen in carrier dropped after reaction start to 140 µM and continuously reduced, until it matched concentration from bulk at 80 µM (fig. 17 B).

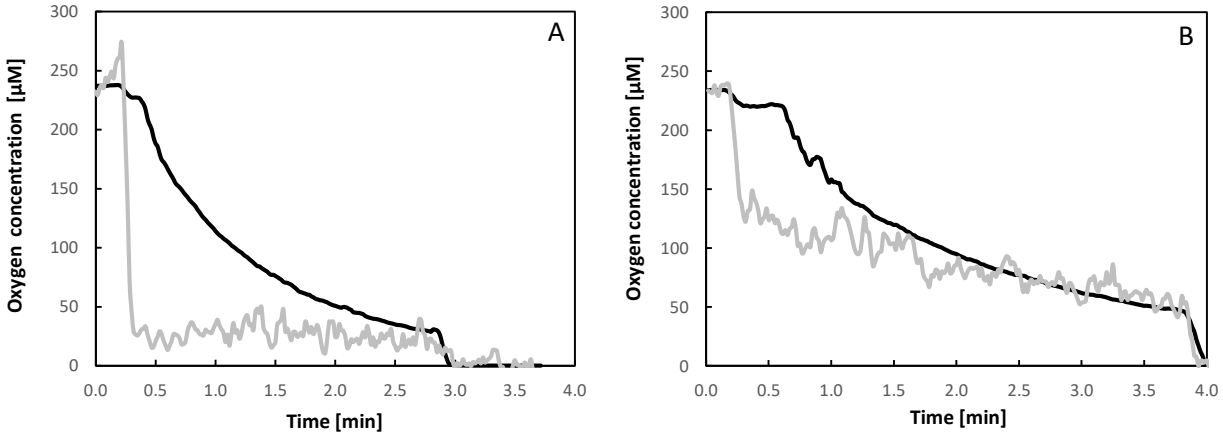

Figure 17: *Oxygen Depletion in bulk and carrier of a loading of 300 U/g A.) Immobilized GOX and catalase B.) Immobilized GOX, hemoglobin and catalase*. Oxygen concentration in carrier measured by luminescent intraparticle measurement. The black line shows oxygen concentration in bulk and grey line shows oxygen concentration in carrier.

The gap between oxygen concentration in bulk and carrier displays mass-transport limitations into particles for given GOX loading. Substrates is transformed faster than it diffuses from bulk into carrier. This means, that regardless how high substrate concentration in bulk, substrate concentration is always limiting for immobilized enzyme, even with immobilized catalase in direct proximity to enzyme. The presence of hemoglobin diminished this gap and improved oxygen supply in carrier significantly. Additionally, oxygen depletion in bulk was also reduced.

The reaction with 30 U/g loading displays an immediate oxygen drop to 140 µM inside of particles after start. After this, oxygen depletion in particles showed the same activity as in bulk (18 A). Apparent activity measured in bulk was 13 U/g. When combined with hemoglobin, oxygen depletion in bulk and carrier matched each other during reaction and no oxygen concentration gap occurred. Apparent activity yin bulk was reduced to 10 U/g (fig. 18 B).

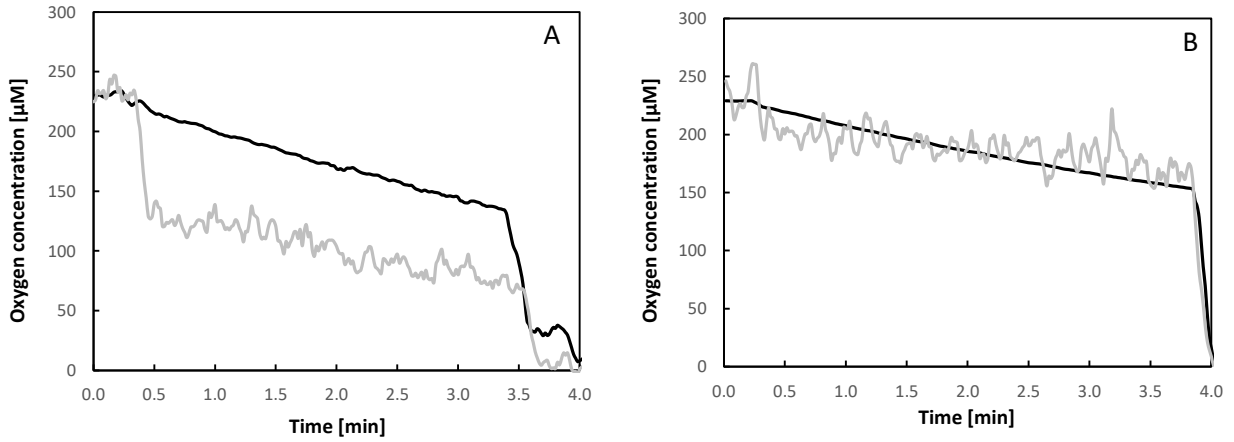

Figure 18: *Oxygen depletion in bulk and carrier of a loading of 30 U/g A.) Immobilized GOX and catalase B.) Immobilized GOX, hemoglobin and catalase*. Oxygen concentration in carrier measured by luminescent intraparticle measurement. The black line shows oxygen concentration in bulk and grey line shows oxygen concentration in carrier.

Mass transport limitations of low loadings were less severe than for high loadings. Oxygen concentration in particles with 30 U/g was lowered by 100 µM, but not reduced to zero. Hemoglobin improved oxygen supply and mass-transport limitations to an extend that oxygen gap between particles and carrier was closed. The co-immobilization of GOX with hemoglobin eliminated mass-diffusion limitation into the porous carrier and supplied GOX with the same concentration of oxygen as in bulk during reaction.

3.3.4 Conclusions

Influence on oxygen depletion in bulk

Oxygen depletion in bulk of slow reactions was significantly reduced by immobilized hemoglobin. This effect declined with when loadings of GOX increases. This could indicate less efficiency of hemoglobin due to higher oxygen consumption of reaction, compared to oxygen storage of hemoglobin. This results and the proof of oxygen release by hemoglobin in oxygen unsaturated solution confirmed the theory of hemoglobin as a possible oxygen vector.

Dissolved hemoglobin on the other hand always led to faster oxygen depletion, indicating a different function regarding oxygen transport than immobilized hemoglobin. This was unexpected. The hypothesis was that dissolved hemoglobin would lead to less substrate imitation in bulk. This could not be fully confirmed by results. To determine if resulting increased usage of oxygen in bulk was beneficial for GOX activity, a comparison with product formation was necessary.

Oxygen depletion in reactions with immobilized GOX and dissolved catalase was clearly lower than without catalase. The presence of hemoglobin had no or just a negligible effect on oxygen depletion combined with dissolved catalase. The immobilization of catalase on the other hand showed mixed results. Reaction had a significantly decreased oxygen depletion if hemoglobin and/or catalase was implemented, except for GOX in combination with immobilized hemoglobin and catalase. Similar to reactions with increased oxygen depletion by dissolved hemoglobin, this led to lower oxygen depletion. There has been no explanation for this phenomenon so far.

Influence on oxygen depletion in carrier

The intraparticle measurement gave an amazing insight in the reaction inside of particles. Reduced oxygen depletion in bulk, induced by immobilized hemoglobin correlated with higher oxygen concentration in carrier. The oxygen concentration gap in- and outside of carrier caused by mass-transport limitations was significantly reduced. In reactions with a low loading of 30 U/g, the gap between bulk and carrier was completely closed.

The gain of information that can be achieved by measurement of oxygen concentration is limited. Activity of GOX is masked by oxygen manipulation, which creates the problem of elucidating the product formation. The peroxide degradation of hemoglobin during reaction eliminates the use of peroxidase assay, which is the usual way to determine product formation of GOX. Instead, the concentration of gluconic acid had to be measured to allow to compare the influence of hemoglobin in product.

3.4. Effect of hemoglobin on productivity

The gluconic acid concentration of reaction with and without hemoglobin was compared to determine the effect on productivity.

The previous experiments concentrated on determination of oxygen depletion as initial reaction rate IRR to determine the influence of hem and catalase. To elucidate the effect of hemoglobin on product formation, it was necessary to change the set-up of experiment. Gluconic acid was measured per HPLC, but to be able to do that, GA conversion had to be increased to mM range to be detectable.

For this, the methodology was changed from measurement of IRR to GA conversion over time course under continuous oxygen supply, to achieve higher product concentrations. The oxygen input was either approached by surface or bubble aeration in suitable reactor. Longer reaction time means a continuous usage of oxygen and therefore substrate limitation. Lower oxygen concentration in bulk would lead to a different effect on hemoglobin during reaction, depending on hemoglobin, implemented in dissolved or immobilized form with enzyme. Only GOX immobilized on porous carrier was used for this set-up, since CLEAs showed a constant hemoglobin leakage, making them not suitable for HPLC measurement.

3.4.1 Surface aerated batch reactor

The gluconic acid concentration of long time reactions under continuous surface aeration were measured to compare influence of hemoglobin on productivity.

Volumetric activity was set by specific activity immobilized on carrier and mass carrier per ml of reaction (eq. 16).

$$Volumetric\ ativity\ [U * ml^{-1}] = \frac{specific\ activity\ [U * g{-}^{1}\ ] * mass\ carrier\ [mg]}{1000\ [mg * g{-}^{1}\ ] * volume\ reactor\ [ml]}$$

Equation 16

The surface aeration reactor consisted of GOX assay in a 15 ml centrifuge tube with 4 ml of reaction volume and 11 ml of air at atm. Glucose was added to a concentration of 200 mM. Oxygen supply was achieved by inversion on a rotator at 20 rpm. Hemoglobin was implemented, either immobilized or dissolved in bulk.

3.4.1.1 Characterization of substrate limitations of immobilizates

Low loadings

GOX loadings of 2, 4, 8, 20, 40 and 70 U/g were offered to carrier. Samples were taken after 96 h to achieve gluconic acid concentration in mM range. 25 mg/ml carrier was added and a volumetric activity between of 0.03, 0.05, 0.22, 0.45, 0.70 and 0.82 U/ml set.

The results of reactions with low loaded catalyst showed that activity of immobilized enzymes did not increase linear with loading, but suffers repression. Loadings of 10 U/g and below showed a slope of an almost linear increase of product, which eased into a plateau if higher loadings were offered to carrier (fig. 19).

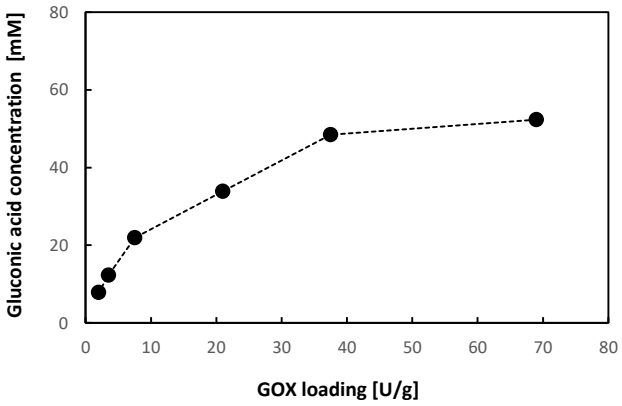

Figure 19*: Gluconic acid concentration of immobilizates with 2, 4, 8, 20, 40 and 70 U/g of GOX loadings after 96 h of reaction at surface aeration. The reaction had volumetric activities of 0.03, 0.05, 0.22, 0.45, 0.70 and 0.82 U/ml.*

Mass-transport limitations led to a leveling of slope. Simultaneously to low oxygen diffusion occurs peroxide accumulation inside of particles, which constantly exposes GOX to high concentrations of peroxide. This are reasons that slope of gluconic acid concentration lessen for increasing GOX loadings and will reach eventually a plateau for certain loadings. The implementation of hemoglobin could, hypothetically, be able to improve the gluconic acid concentration and ideally improve the issue of product plateau for higher loadings by mass-transport improvement.

High loadings

The activity of particles with loadings of 300, 400, 700, 1100, 1600 and 2400 U/g of GOX were measured and 1.25 mg/ml particles added to reaction, to set volumetric activity between 0.38 to 0.029 U/ml. Samples were taken after 7 h.

At loadings this high, product concentration was nearly stagnant. Particles with loadings of 700 U/g and higher reached a plateau (fig. 20).

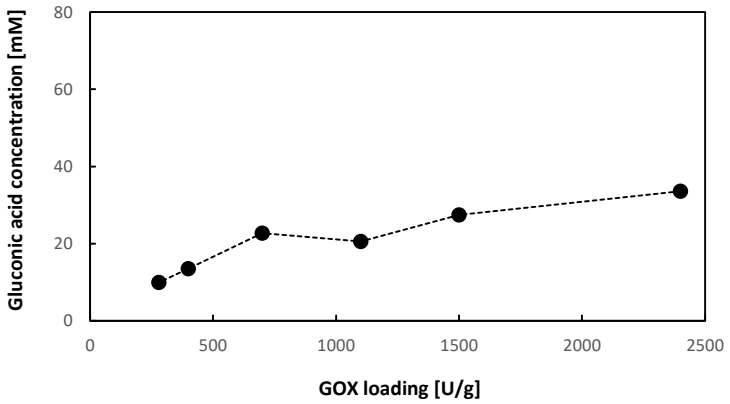

Figure 20: *Gluconic acid concentration of 300, 400, 700, 1100, 1600 and 2400 U/g of loadings after 7 h of reaction at surface aeration. The reaction had a volumetric activity of 0.21, 0.38, 0.20,0.33, 0.35, 0.30 U/ml.*

The loading of biocatalyst determined the mass transport that occurs from liquid into solid phase of carrier. If mass-transport in particle is lower than reaction, this also means peroxide accumulation inside of particles. These are reasons why gluconic acid concentrations of different GOX loadings show a steep slope for low loadings and reach eventually a plateau for increased loadings.

3.4.1.2 Effect of hemoglobin on gluconic acid concentration

Gluconic acid production of low loadings of immobilized GOX and hemoglobin

This experiment was done to study the influence of hemoglobin on gluconic acid production of catalysts with low GOX loadings. This was done to determine the effect of hemoglobin on reactions with low substrate limitations in long time performance of GOX.

2, 4, 8, 20, 40 and 70 U/g of GOX were loaded onto carrier. 25 mg/ml of particles with measured activities of 1.2, 2.0, 8.6, 17.8, 28.0 and 32.6 U/g of GOX were added per reaction, to achieve a volumetric activity between 0.029 to 0.82 U/ml.

Samples were taken after 96 h. Gluconic acid concentration were compared between reactions with hemoglobin implemented either in dissolved or immobilized form with the GOX baseline.

The product concentration of reactions with immobilized hemoglobin were more than doubled compared to GOX baseline and tripled with dissolved hemoglobin (fig. 21). Reactions with immobilized and dissolved hemoglobin showed steeper slope of product concentration for catalysts with loadings under 10 U/g. Reactions with catalyst loadings higher than 20 U/g reached a plateau of product concentration, regardless if hemoglobin was implemented or not.

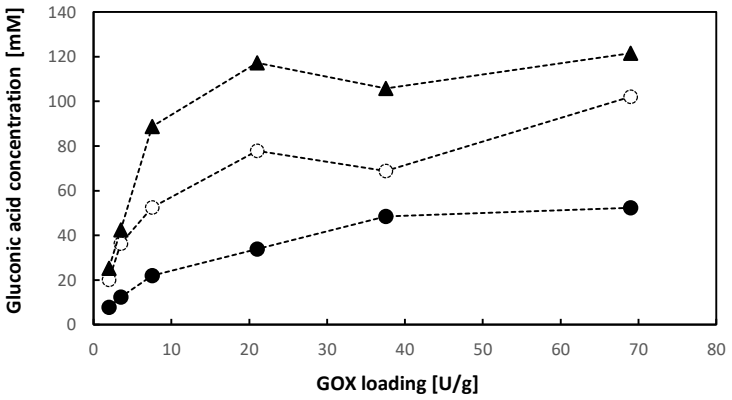

Figure 21*: Gluconic acid concentration of reaction with 2, 4, 8, 20, 40 and 70 U/g of GOX loadings with and without hemoglobin after 96 h. Reaction contained 25 mg/ml of carrier and 2.0 mg/ml of hemoglobin (immobilized or dissolved) with volumetric activities of 0.82, 0.70, 0.45, 0.22, 0.05, 0.03, under continuous surface aeration. Immobilized GOX is shown as •, GOX with immobilized hemoglobin as ○ and GOX with dissolved hemoglobin as ▲.*

The productivity of reaction greatly benefited when hemoglobin was implemented. The dissolved form of hemoglobin had contact with gas-liquid interphase, which was expected to work like a simplified blood circulation system. Hemoglobin in immobilized form had no direct contact with gas-phase. This experimental set-up should show if hemoglobin is able to work as an oxygen vector, when immobilized on carrier. Hemoglobin seems to be able to work as oxygen transport without direct contact with gas interphase. The implementation of hemoglobin seems to boost the productivity, until a certain concentration is reached. The co-substrate glucose was not limiting for reaction, since 80 mM of the start concentration were still left.

The plateau of gluconic acid concentration that arose from increased loadings could be either the effect of substrate limitation into carrier and/or enzyme inactivation by peroxide. The elimination of peroxide during reaction would be necessary to eliminate the damaging effect of peroxide and determine the effect of oxygen limitation into carrier.

Activity measured as initial reaction differs from long time reactions. Usually enzymes suffer inactivation by use and substrate limitation.

The amount of actual produced gluconic acid would therefore be lower than calculated theoretically from initial reaction rate. This calculated percentage defines the efficiency of long-time reactions compared to the initial reaction rate. The ratio of this two concentrations told how efficiency of production changed with implementation of hemoglobin at increasing loadings. In combination with hemoglobin, it showed how substrate limited reaction was improved. For the calculation of theoretical gluconic acid concentration the IRR of immobilized GOX and reactor volume was used, since the presence of hemoglobin disguised oxygen depletion (equ.17).

$$Product\ concentration\ [mM] = \frac{specific\ activity\ [U * g^{-1}] * mass\ carrier\ [mg] * 60\ [min * h^{-1}] * 1000\ [ml * l^{-1}]}{volume_{reactor}\ [ml] * 1000\ [mg * g^{-1}] * 1000\ [\mu mol * mmol^{-1}]}$$

Equation 17

The productivity decreased with increasing GOX loadings. The ratio of GOX mono immobilizate was over 50 % for loadings under 10 U/g showed an efficient GA production in reactions with immobilized GOX of approx. 50 % of theoretical concentration. When hemoglobin was present, the productivity even reached 120 to 160 % of possible product concentration. The productivity dropped very fast for loadings over 20 U/g while the use of hemoglobin had less influence (fig. 22).

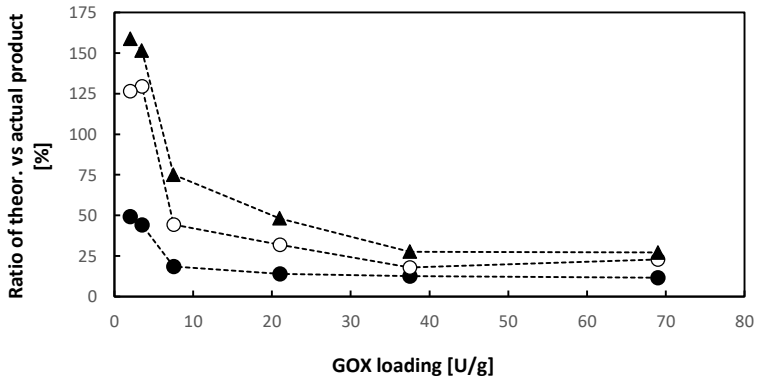

Figure 22: *Ratio of product formation over long time vs initial reaction rate of different GOX loadings of reaction with 25 mg/ml and 2.0 mg/ml of hemoglobin, after 96 h at surface aeration. Reactions with volumetric activities of 0.03, 0.05, 0.22, 0.45, 0.70 and 0.82 U/ml. Immobilized GOX is shown as ●, GOX with immobilized hemoglobin as ○ and GOX with dissolved hemoglobin as ▲.*

Compared to IRR measured, productivity of GOX mono immobilizate was lowered, which is usual for long time reactions. The efficiency of reaction could be significantly improved by implementation and even succeeded the IRR for very low loadings. The effect diminished, the higher the loading. This experiment showed the effect of hemoglobin at low mass transport limitations into carrier.

The suitability of hemoglobin as oxygen vector in reaction with high mass-transport limitations at similar volumetric activity was the next step to be tested.

Gluconic acid production of high loadings of immobilized GOX and hemoglobin

GOX loadings of 300, 400, 700, 1100, 1600 and 2400 U/g were immobilized and a specific activity of 239, 280, 266, 161, 304 and 169 U/g measured. 1.25 mg/ml particles were added to reaction, to achieve a volumetric activity between 0.38 to 0.029 U/ml.

0.1 mg of hemoglobin per ml of reaction volume were implemented, either immobilized or dissolved in bulk. Samples were taken after 7 h.

Despite the high loadings, the addition of dissolved hemoglobin lead to almost 3-fold increase of product concentration and a slightly underperformance for immobilized hemoglobin compared to GOX baseline. The productivity of immobilizates showed a plateau for all loadings (fig. 23).

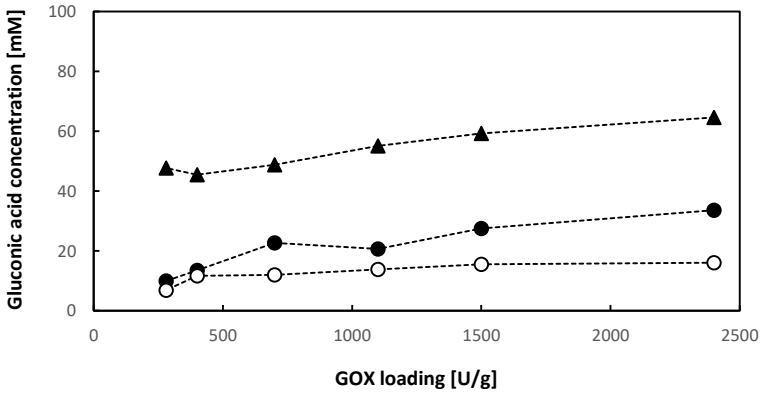

Figure 23*: Comparison of gluconic acid concentration of reaction with 300, 400, 700, 1100, 1600 and 2400 U/g of GOX loadings with dissolved or immobilized hemoglobin after 7 h of reaction. Reaction contained 1.25 mg/ml of carrier and 0.1 mg/ml of hemoglobin with volumetric activities of 0.21, 0.38, 0.20,0.33, 0.35, 0.30 U/ml, under continuous surface aeration. Immobilized GOX is shown as •, GOX with immobilized hemoglobin as ○ and GOX with dissolved hemoglobin as ▲.*

At gluconic acid concentrations of 20- 120 mM, the same concentrations of peroxide were produced. The influence of hem on substrate limitation for dissolved hemoglobin was clearly visible, while immobilized hemoglobin seemed to be prone to change of conditions or disturbing factors.

All immobilizates showed a plateau of gluconic acid concentration despite the increase in total product by after addition of hemoglobin. Apart from mass-transport limitations of product, the accumulation of peroxide inside of particles due to the same mass-transport problem probably lead to enzyme inactivation over time.

This could possibly explain why the samples with immobilized hemoglobin didn´t show an increase of product compared to the experiment before. While effect of peroxide might be negligible for GOX during initial reaction rate, it is very likely a reason for inactivation of GOX due to high concentration and longtime exposure.

The ratio of theoretical product concentration vs actual product concentration for the GOX baseline and reaction with immobilized hemoglobin laid between 5 and 20 %, between 20 and 35 % for reactions with dissolved hemoglobin (fig 24). The ratios were significant lower than for lower particle loadings, even if the reactions with higher loadings were set to lower volumetric activity in reactor.

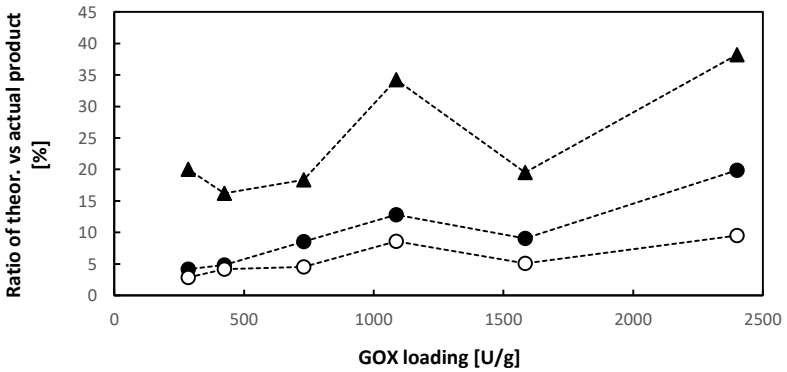

Figure 24*: Ratio of product formation over long time vs initial reaction rate of 300, 400, 700, 1100, 1600 and 2400 U/g of GOX loadings of reaction with 1.25 mg/ml of carrier and 0.1 mg/ml of hemoglobin and volumetric activities of 0.21, 0.38, 0.20, 0.33, 0.35, 0.30 U/ml after 7 h under continuous surface aeration. Immobilized GOX is shown as •, GOX with immobilized hemoglobin as ○ and GOX with dissolved hemoglobin as ▲.*

The implementation of dissolved hemoglobin lead to a clear increase of this ratio. This showed that the efficiency of reaction can be improved for even very high loadings.

The ratio of theoretical and actual product showed that the efficiency of long time reactions dropped significantly for high enzyme loadings. Mass-transport limitations, that lead to oxygen limitation in particles, are also responsible for peroxide accumulation. The high peroxide concentration that occurred during reaction, indicated by product plateau, sparked the question of a possible degradation or inactivation of hemoglobin.

In the previous chapter of measurement of oxygen depletion, the peroxidase activity of hemoglobin was measured, leading to change of oxidation state of hemoglobin. Literature research showed that during peroxide degradation, ferrous human hemoglobin (Fe^{2+}) is converted to ferryl state (Fe^{4+}) which rapidly autoreduces back to the ferric form (Fe^{3+}), creating a hydroxyl radical and a hydroxide ion. Degradation of a second peroxide molecule lead to re-reduction of heme from ferric to ferrous state, by production of a superoxide ion.

Hydroxyl radical from the first reaction is reduced to hydroxide ion and superoxide ion is oxidized to singlet oxygen (fig. 25.), [32], [41], [42].

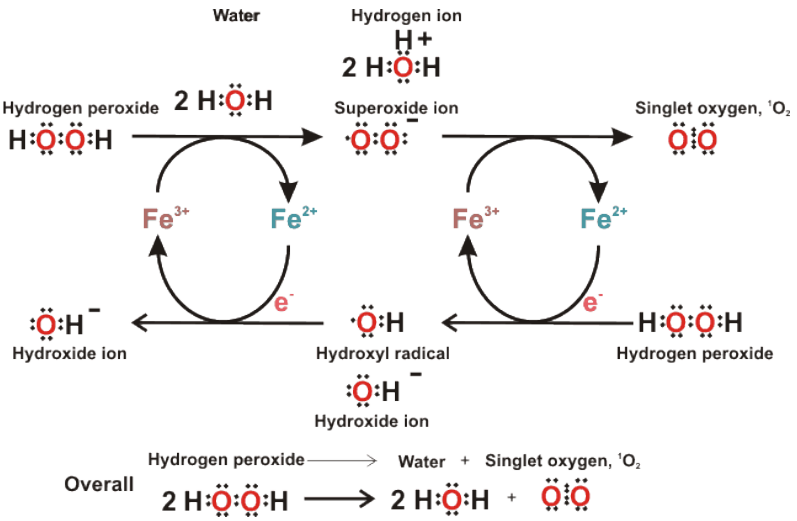

Figure 25: *Degradation of peroxide by hemoglobin heme in two linked cycles. Peroxide degradation leads to conversion of ferrous human hemoglobin (Fe^{2+}) to ferryl state (Fe^{4+}) which rapidly autoreduces back to the ferric form (Fe^{3+}), creating a hydroxyl radical and a hydroxide ion. Degradation of a second peroxide molecule lead to re-reduction of heme from ferric to ferrous state, by production of a superoxide ion. Hydroxyl radical from the first reaction is reduced to hydroxide ion and superoxide ion is oxidized to singlet oxygen* [32].

With this ability, hemoglobin works as a type of enzyme, by being able to degrade more peroxide than active side offered of hemoglobin. Frontier et al. however, state that in the presence of H2O2 the ferric state of hemoglobin can form both ferryl hemoglobin and a protein radical in a "pseudoperoxidative cycle", that results in heme degradation [43]. This indicates that hemoglobin can spontaneously attack glucose oxidase if in ferrous state and peroxide is present. Other literature confirmed the autoxidation modus of hemoglobin, resulting in protein radicals and chain reactions [44]. The elimination of peroxide during reaction was another attempt to prevent degradation of hemoglobin and GOX radical building by implementation of catalase into reaction.

3.4.1.3 Effect of catalase and hemoglobin on reaction

The bovine liver catalase was applied in reaction to provide some important benefits for the reaction. The catalase degrades one mol of peroxide and generates ½ mol oxygen and 1 mol of water. The enzyme is very effective and fast. Catalase was used to prevent the accumulation of peroxide and for the protection of GOX and hemoglobin during reaction, as well as for oxygen recycling during reaction to improve substrate limitation during reaction.

The implementation of catalase can be distinguished by implementation of dissolved catalase and immobilized catalase. Dissolved catalase can be applied in excess, but has no proximity to enzyme and immobilized hemoglobin. The immobilization of catalase further means a loss of enzyme, and eventually activity loss.

Influence of dissolved catalase and hemoglobin on gluconic acid production

It was studied how catalase improved the effect of hemoglobin. The net effect of catalase on reaction was determined by comparison of reaction by using GOX with and without catalase. For the experiment with dissolved catalase, loadings of 80, 150 and 350 U/g of GOX were offered to porous particles and immobilized. Hemoglobin implemented in dissolved or immobilized form. 25 mg/ml of carrier were added to the reactor to achieve volumetric activity of 4.3, 1.5 and 0.95 U/ml at a hemoglobin concentration of 2 mg/ml. Samples were taken after 3 hours.

The immobilizates with dissolved hemoglobin showed the usual improvement (fig. 26 A). Immobilized hemoglobin led to a slightly underperformance compared to the GOX baseline.

The addition of catalase led to an improvement of productivity of every immobilizate (fig. 26 B). The combination of GOX with dissolved catalase (GOXCAT) had a higher productivity than the immobilizate with GOX, immobilized hemoglobin and catalase (GOXHEMiCAT), but was still lower than for GOX, dissolved hemoglobin and catalase (GOXHEMdCAT). The pure GOX immobilizates showed the highest benefit by dissolved catalase but also a productivity plateau for increasing loadings between 25 to 30 mM compared to immobilizates with hemoglobin and catalase, which showed increasing gluconic acid concentrations.

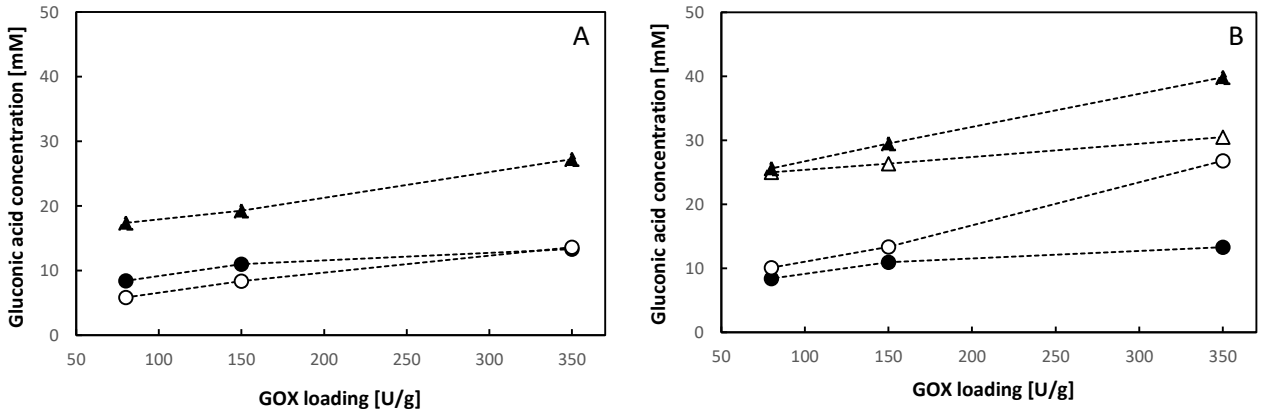

Figure 26*: Comparison of gluconic acid concentration of 80, 150 and 350 U/g GOX loadings and hemoglobin in reactions with and without dissolved catalase A.) Immobilized GOX is shown as •, GOX with immobilized hemoglobin as ○ and GOX with dissolved hemoglobin as ▲. B.) Immobilized GOX is shown as •, GOX with catalase as Δ, GOX with immobilized hemoglobin and catalase as ○ and GOX with dissolved hemoglobin and catalase as ▲. Reaction in 4 ml 50 mM PBB, 200 mM glucose, 25 mg of carrier and 2 mg of hemoglobin per ml of reaction and a volumetric activity of 3.73, 1.15 and 0.6 U/ml, under continuous surface aeration by inversion.*

The implementation of dissolved catalase led always to an improved productivity for loadings of 350 U/g of GOX. The pure GOX immobilizate showed the highest benefit from implementation of dissolved catalase but the gluconic acid concentration of the immobilizate with GOX, dissolved hemoglobin and catalase (GOXHEMdCAT) was the highest.

The bovine liver catalase is a very efficient enzyme and was added to reaction in excess and made the peroxidase activity of hemoglobin on productivity, determined in the previous chapter of hemoglobin characterization, negligible. The additional increasing effect of hemoglobin on gluconic acid production was therefore supposedly not the effect of peroxidase effect of hemoglobin but due to higher oxygen supply.

The amount of actual produced gluconic acid was again compared to theoretically activity, calculated from initial reaction rate. This calculated percentage defines the efficiency of long-time reactions compared to the initial reaction rate. (equ.17).

The ratio of GOX with dissolved hemoglobin started at 25 % and decreased to ~5 %. Ratio of GOX baseline and with immobilized hemoglobin was under 10 % and dropped below 4 % (fig. 27A). By implementation of catalase, the ratio of GOX immobilizate and the immobilizate with dissolved hemoglobin was increased to ~25 % of efficiency. The combination of catalase and immobilized hemoglobin showed only an insignificant increase (fig. 27 B).

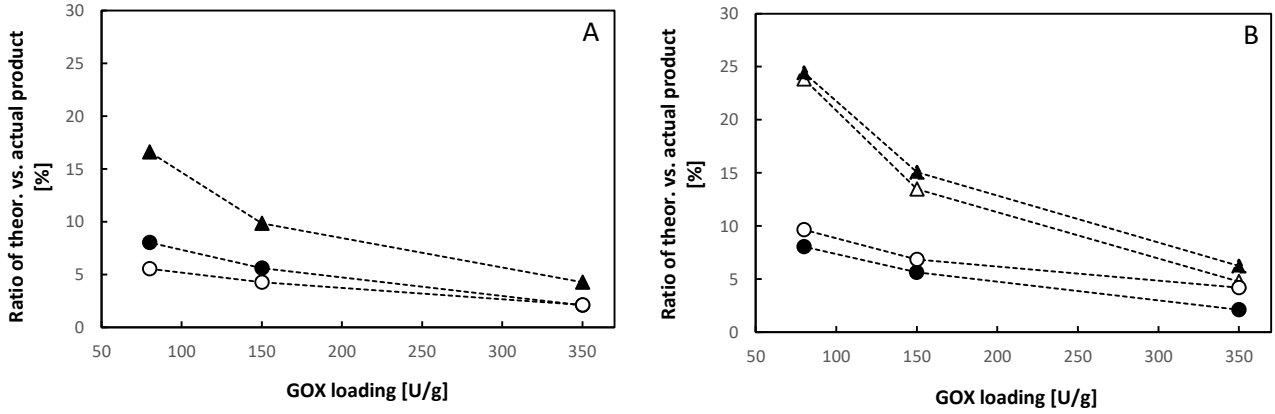

Figure 27*: Ratio of actual product concentration to theoretical product concentration of reaction with 350, 150 and 80 U/g GOX loadings and hemoglobin in reaction with and without dissolved catalase. Reaction in GOX assay with 25 mg/ml of carrier and 2.0 mg/ml of hemoglobin per ml and a volumetric activity of 3.73, 1.15 and 0.6 U/ml in surface aeration reactor A.) Immobilized GOX is shown as •, GOX with immobilized hemoglobin as ○ and GOX with dissolved hemoglobin as ▲. B.) Immobilized GOX is shown as •, GOX with catalase as Δ, GOX with immobilized hemoglobin and catalase as ○ and GOX with dissolved hemoglobin and catalase as ▲.*

The ratio of actual product concentration vs theoretical product concentration was notable reduced due to higher GOX loadings. The substrate limitation into carrier, caused by higher loadings, and peroxide exposure during reaction, were the main criterion in performance deterioration. Samples containing catalase always performed better. The GOX mono immobilizate and GOXHEMd showed the highest benefit from implementation of dissolved catalase. The ratio of GOXHEMiCAT did not show any significant improvement. The effect of co-immobilization of catalase with GOX and hemoglobin was to be studied in the next experiment for its beneficial effect compared to dissolved catalase.

Influence of immobilized catalase and hemoglobin on gluconic acid production

The immobilization of catalase was done to study the benefit of peroxide degradation in reactions with GOX and immobilized or dissolved hemoglobin. The net effect of catalase was determined by comparison of reaction with GOX baseline and GOX with catalase.

Particles with immobilized GOX, catalase and hemoglobin implemented in immobilized or dissolved form were prepared and 200 U/g of GOX loading, ~75,000 U/g (22 mg/g) of catalase and 100 mg/g of hemoglobin were offered to carrier. The activity of immobilized GOX was measured to be 83 U/g and 2.5 mg of carrier per ml added to reaction, to achieve a volumetric activity of 0.21 U/ml (equ. 16) and with 0.2 mg/ml of hemoglobin in reactor. The reaction was carried out for 6 h every hour to show how oxygen recycling and protection influenced reaction over time. The implementation of immobilized and dissolved hemoglobin without catalase caused a clear increase of product concentration compared to GOX baseline (fig. 36 A). Reactions with dissolved hemoglobin performed again better by almost 3-fold increase of product concentration after 6 h, while reactions with immobilized hemoglobin showed a clear increase. For particles with immobilized GOX and hemoglobin it was noticeable that product concentration started very similar for the GOX baseline at the beginning and developed a steeper slope of product concentration over time. Immobilized GOX and catalase showed no significant increase of gluconic acid concentration.

The benefit of catalase compared to hemoglobin or in dissolved state was negligible (fig. 28 B). The gluconic acid produced by immobilized hemoglobin and catalase was increased from 14 mM to 30 mM after 6 hours. (fig. 28 B). Gluconic acid of immobilizates with dissolved hemoglobin and catalase increased from 33 to 40 mM.

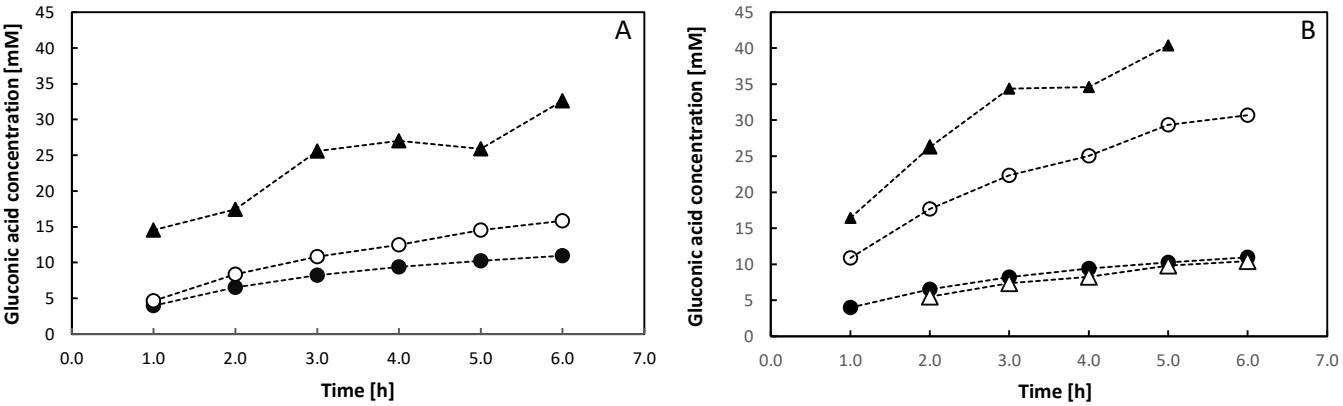

Figure 28*:* Comparison of *gluconic acid concentration of 200 U/g GOX loading and hemoglobin, in reactions with and without catalase for 6 h. Reaction in GOX assay, with 2.5 mg of carrier and 0.2 mg of hemoglobin per ml of reaction and a volumetric activity of 0.21 U/ml under continuous surface aeration. A.) Immobilized GOX is shown as •, GOX with immobilized hemoglobin as ○ and GOX with dissolved hemoglobin as ▲. B.) Immobilized GOX is shown as •, GOX with catalase as Δ, GOX with immobilized hemoglobin and catalase as ○ and GOX with dissolved hemoglobin and catalase as ▲. Reaction in GOX assay, with 2.5 mg of carrier and 0.2 mg of hemoglobin per ml of reaction and a volumetric activity of 0.21 U/ml under continuous surface aeration.*

The benefit of catalase addition was highest for samples with immobilized hemoglobin, while dissolved hemoglobin only showed a slight improvement. When catalase was implemented, immobilized hemoglobin caused a steeper slope of product concentration and the difference to GOX baseline amplified over time.

The ratio of theoretical vs actual gluconic acid concentration of GOX baseline without catalase started at ~35% and decreased to 20 %. The implementation of hemoglobin showed a higher ratio than GOX baseline when immobilized and an even higher ratio for dissolved hemoglobin (fig. 29 A). If hemoglobin was combined with catalase, the ratio increased to 100 % (immobilized hemoglobin) and almost 140 % (dissolved hemoglobin) after the first hour of reaction (fig. 29 B). After 6 h the efficiency decreases significantly. The highest benefit of hemoglobin and catalase is in the early phase of reaction and draw near the GOX baseline with progressing reaction.

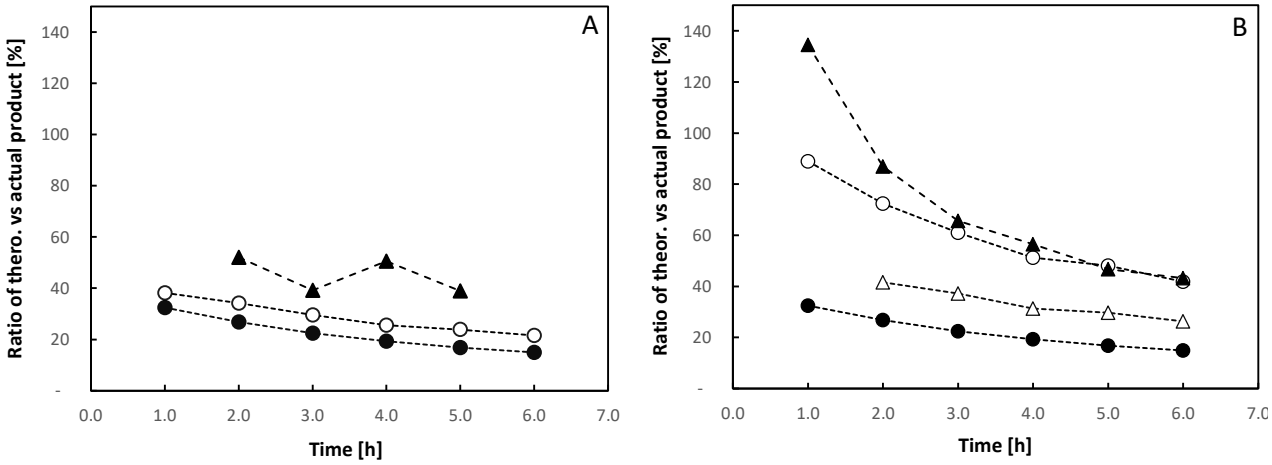

Figure 29: *Ratio of theoretical product concentration to actual product concentration gluconic acid concentration of reaction with 200 U/g GOX loading, hemoglobin with and without catalase for 6 h. Reaction in GOX assay 25 mg/ml of carrier and 2.0 mg/ml of hemoglobin with volumetric activity of 0.21 U/ml. A.) Immobilized GOX is shown as ●, GOX with immobilized hemoglobin as ○ and GOX with dissolved hemoglobin as ▲. B.) Immobilized GOX is shown as ●, GOX with catalase as Δ, GOX with immobilized hemoglobin and catalase as ○ and GOX with dissolved hemoglobin and catalase as ▲.*

The combination of immobilized hemoglobin with catalase made the reaction very effective for the loading of 200 U/g GOX, causing to reach concentrations almost as high as calculated from initial reaction rate after the first hour. Dissolved hemoglobin and catalase lead to gluconic acid concentrations higher than the calculated theoretical product concentration, but this efficiency deteriorated quickly after some hours. The effect of catalase and hemoglobin decreased massively over time and got more similar to GOX baseline.

To summarize the effect of catalase implementation; dissolved catalase had no direct proximity to enzyme and had no direct influence on mass-transport limitation apart from oxygen recycling in bulk. This led to a higher oxygen concentration in bulk, which results in a higher oxygen gradient compared to reactions without catalase.

Mass-transport limitations also lead to peroxide accumulation inside of carrier, making peroxide degradation by dissolved catalase less efficient. Therefore, the activity of peroxide degradation was not dictated by activity of catalase, but by peroxide diffusion rate into bulk, while GOX was suffering from inactivation by high concentrations in carrier.

The amount of catalase that can be immobilized on the other side is limited and its activity can be impaired by the immobilization process. The direct proximity of catalase to enzyme and hemoglobin should prevent inactivation and oxidation by peroxide accumulation. The effect of oxygen supply by immobilized catalase was however small, since the effect of oxygen recycling in carrier was dictated by a small oxygen-reservoir due to low volume inside of carrier.

3.4.1.3 Conclusions

The implementation of hemoglobin can help to improve the performance of immobilized GOX. While dissolved hemoglobin was always more effective, the immobilization of hemoglobin had a lot of advantages like easy separation from product.

The benefit of immobilized hemoglobin was expected to be its ability to release oxygen at decreasing partial pressure. The concentration of hemoglobin implemented in reaction could hold and release a maximum of 123 µM of oxygen, which poses a third of concentration of bulk. The benefit of hemoglobin during IRR reaction rate is mainly due to oxygen release during reaction.

In a reactor with conversion over time, oxygen drops to limiting concentration after few minutes and remains limiting. This means hemoglobin would release oxygen once. In a conversion with mM product range, 123 µM of oxygen is an insignificant input. This indicated a further effect for immobilized hemoglobin, such as re-uptake of oxygen for dissolved hemoglobin from gas-liquid interphase or even a pull of oxygen into liquid phase without contact with gas phase.

The use of catalase was very beneficial for the gluconic acid production and improved reactions with hemoglobin by more than just its net effect. Both implementation methods of catalase had different benefits and drawbacks, dependent on limitation in carrier (mass-transport limitation by high enzyme loadings) and oxygen limitation in bulk due to volumetric activity (volume -oxygen reservoir) of reaction set-up. The immobilization of catalase was more beneficial for carrier with immobilized hemoglobin and low loadings due to peroxide degradation. Hemoglobin and GOX were protected from increasing peroxide concentrations by catalase. The experiments so far have

shown the influence of hemoglobin and catalase in reactions with depleting oxygen concentration but without simultaneously oxygen measurement.

By now the results confirmed the positive effect of hemoglobin on reactions of decreasing oxygen concentration. To gain more information a method change was done to implement hemoglobin in reactions with fixed oxygen concentrations, leading to different oxygen gradients into particles.

3.4.2 Bubble aeration batch reactor

The effect of hemoglobin in reactions of fixed oxygen concentrations was studied by comparing gluconic acid production of co-immobilized GOX and catalase (GOXCAT) to co-immobilized GOX, catalase and hemoglobin (GOXHEMCAT).

Three different set-ups of oxygen concentration in liquid phase were chosen as reactor runs with 200 µM, 100 µM or 50 µM of oxygen (fig. 30), setting different non-limiting or limiting concentrations in bulk. This means that previous reactions of uncontrolled oxygen depletion were "fragmented" into different set-ups of oxygen concentration, to determine the effect of hemoglobin at different levels of oxygen supply.

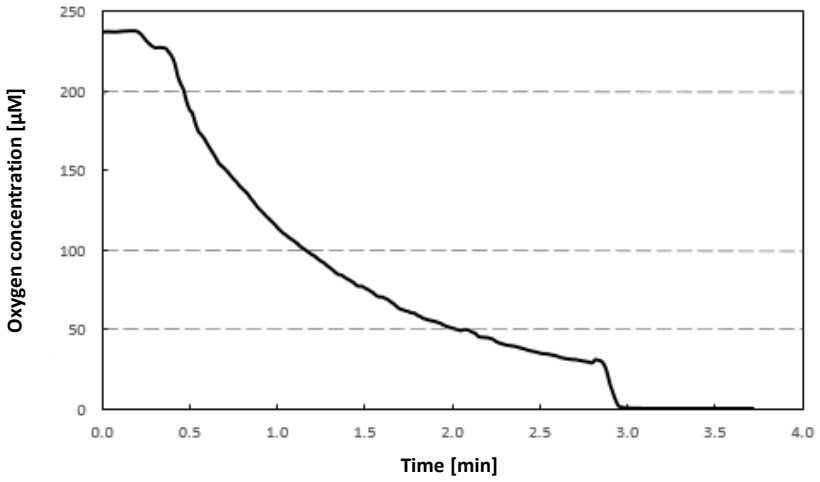

Figure 30*: Partitioning of reaction with GOX into different stages of substrate supply by bulk. The figure shows different oxygen concentrations of 200, 100 and 50 µM in bulk in a reaction without aeration. Each concentration in bulk causes different oxygen gradients into particles for the same reaction rate inside the particle.*

With fixed oxygen concentrations in bulk, certain oxygen gradients into particles occur during reaction. The higher the oxygen difference of the gradient, the higher the oxygen-transfer rate (OTR) into particle [45]. If oxygen consumption in particle is higher than OTR, oxygen in particle is used up quickly and oxygen concentration remains low or even zero µM.

The bubble aeration allowed a continuous oxygen monitoring during long time reactions to manually control the oxygen concentration in bulk. As a pre-calculation for needed materials of reactor runs, 150 U/g of GOX loading were chosen, of which 46 U/g are approximately immobilized (measured in previous experiment of middle high loadings). The particle mass in a 50 ml reactor volume with given loading was to be prepared, to achieve a desired productivity of approximately 80 mM/h (or 1.3 µmol/min/ml) of gluconic acid. Mass carrier was calculated as 1.46 g of carrier, based on the empirical value of 46 U/g of apparent activity (equ. 18).

$$1.46\ [g_{carrier}] = \frac{80\ [mmol * h^{-1} * l^{-1}] * 0.05\ [l] * 1000\ [\mu mol * mmol^{-1}]}{46\ [\mu mol * min^{-1} * g^{-1}] * 60\ [min * h^{-1}]}$$

Equation 18

3.4.2.1 Bubble Reactor with constant oxygen concentration of 200 µM

At this non-limiting condition in bulk, substrate limitation only occurs by mass-transport limitations into carrier. This set-up is comparable to initial reaction phase of oxygen depletion during non-aerated reactions. This shows the influence on hemoglobin and its oxygen release during reaction at fixed high oxygen concentration in bulk.

For the reactor run 170 U/g of GOX was offered to particles and an apparent activity of 67 U/g measured. Afterwards 22 mg of catalase per g and 100 mg/g of hemoglobin were offered to carrier. To be able to saturate the bulk at 200 µM, with the given aerated reactor (see experimental section) the mass of carrier was reduced by half for this reactor run and 0.73 g of carrier added to reactor (14.6 mg/ml). The productivity of the prepared catalyst was calculated as a 58.7 mM GA per hour, based on actual measured apparent activity (equ. 19).

$$58.7\ [mmol * h^{-1} * l^{-1}] = \frac{0.73\ [\mathrm{g}]\ *\ 67\ [\mu\mathrm{mol} * \mathrm{min}^{-1} * \mathrm{g}^{-1}] *\ 60\ [\mathrm{min} * \mathrm{h}^{-1}]}{0.05\ [\mathrm{l}] *\ 1000\ [\mu\mathrm{mol} * \mathrm{mmol}^{-1}]}$$

Equation 19

The reaction contained 1.17 mg/ml of immobilized hemoglobin and was started by addition of glucose to a concentration of 200 mM. When oxygen depletion was measured, the GOXCAT immobilizates had an oxygen depletion of 0.39 U/ml and the GOXHEMCAT immobilizate 0.31 U/ml. When oxygen concentration reached a highly limiting concentration of 30 µM, the bubble

aeration was started. A range of +/-20 µM of oxygen was tolerated. Samples were taken every half hour for 3 h and every second hour until 9 h and the last taken after 23 h. The oxygen concentration was manually controlled for 6 hours and afterwards left for additional 18 h at fixed flow.

At steady state of oxygen input and consumption, both reactor runs showed an oscillating oxygen concentration. The GOXCAT immobilizate showed a saturated oxygen concentration of 250 µM after 10 h while the GOXHEMCAT immobilizate reached oxygen saturation after 14 h (fig. 31).

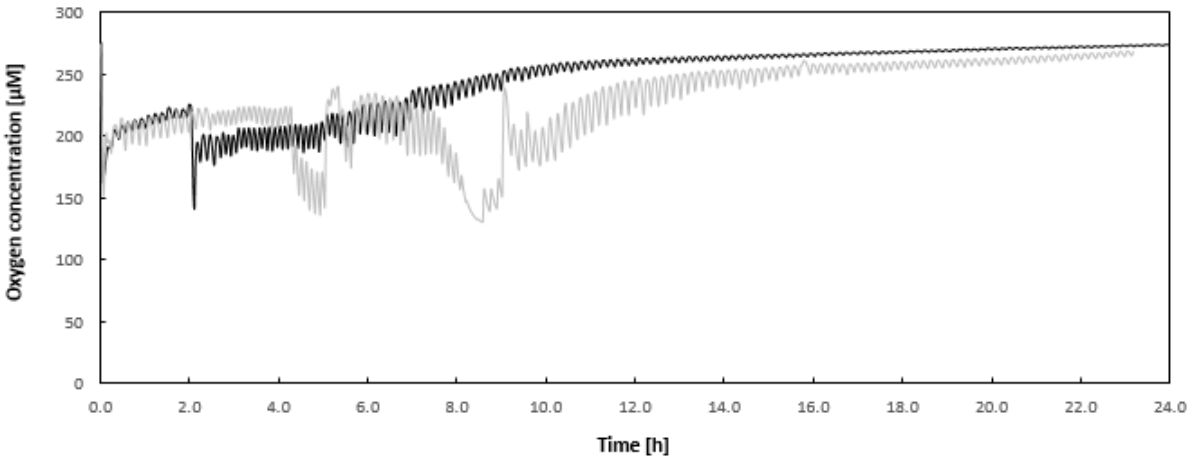

Figure 31*: Comparison of oxygen concentration fluctuation in a 50 ml reactor with immobilized GOX and catalase with and without hemoglobin at continuous bubble aeration, manually set to 200 µM. Oxygen depletion of the GOX and catalase co-immobilizate was 0.39 U/ml and 0.31 U/ml for the immobilizate with GOX, catalase and hemoglobin. The black line indicates the GOX catalyst with catalase and the gray line the GOX, hemoglobin and catalase catalyst.*

Oxygen saturation for both reactor runs were comparable with a similar supply of 200 µM oxygen for both catalysts, GOXCAT and GOXHEMCAT. Therefore, all samples were comparable for evaluation. After 9 h enzyme activity decreased so much that, the oxygen input of flow exceeded oxygen consumption of enzymes, causing the bulk to saturate with oxygen.

The GOXCAT immobilizate showed higher gluconic acid concentrations than the GOXHEMCAT immobilizate. The increase of product showed a similar slope for both catalysts. While oxygen concentration increased due to loss of enzymatic activity after 9 h, gluconic acid of immobilizates still increased from 17 mM to 25 mM (GOX, hemoglobin and catalase) and 19 mM to 32 mM (GOX and catalase) after 9 h of reaction (fig. 32).

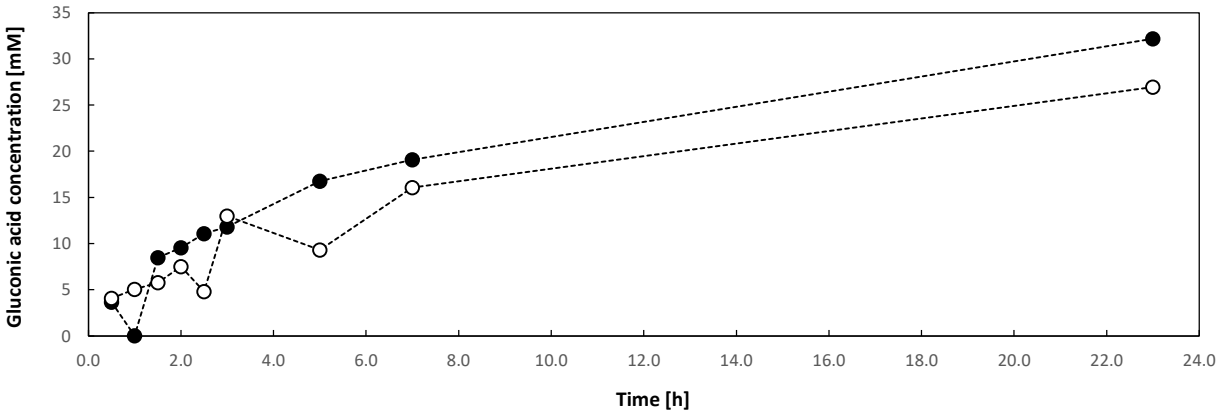

Figure 32*: Comparison of gluconic acid concentration over time of immobilized GOX and catalase with and without hemoglobin in bubble aeration reactor with 200 µM of oxygen concentration. Oxygen depletion of the GOX and catalase co-immobilizate was 0.39 U/ml and 0.31 U/ml for the immobilizate with GOX, catalase and hemoglobin. The GOX and catalase catalyst is shown as • and the GOX, hemoglobin catalase catalyst as ○.*

After 9 h the particles showed severe signs of mechanical degradation and the enzymatic activity was reduced so much, that oxygen consumption was lower than oxygen input by aeration. The visible degradation of particles parallel to increase of product sparked the assumption if enzyme was released from particle and conducted the reaction as dissolved enzyme, leading to fading activity and mass transport limitations of reaction over time. If GOX of catalysts was gradually released to bulk at constant oxygen supply of 200 µM, substrate limitation is not given and any effect of hemoglobin was non-existent. Another reason could be the autoxidation of hemoglobin by oxygen supply, leading to the production of oxygen radicals and damage of GOX (fig. 33). This was to be determined by the next reactor runs at lower oxygen concentrations. Since the gluconic acid concentration of both reactor runs were very similar, the underperformance of GOXHEMCAT immobilizate could have also occurred from gluconic acid imbalance of HPLC measurement.

The results showed no explicit benefit of hemoglobin at a constant oxygen concentration of 200 µM in the reactor. It was not identified if the benefit of hemoglobin did not work at the non-limiting conditions in bulk or the conditions of the bubble reactor lead to this results. In the next

experiment the influence of hemoglobin at non saturated concentration of 100 µM of oxygen was tested for comparison.

3.4.2.2 Bubble Reactor with continuous oxygen concentration at 100 µM

160 U/g of GOX were offered to carrier and an apparent activity of 63 U/g measured. Hemoglobin and catalase were immobilized as usual. 1.46 g of carrier for 50 ml were prepared, which would result in a productivity of approximately 110.4 mM/h (equ. 20).

$$110.4\ [mmol * h^{-1} * l^{-1}] = \frac{1.46\ [\mathrm{g}]\ *\ 63\ [\mu\mathrm{mol} * \mathrm{min}^{-1} * \mathrm{g}^{-1}] *\ 60\ [\mathrm{min} * \mathrm{h}^{-1}]}{0.05\ [\mathrm{l}] *\ 1000\ [\mu\mathrm{mol} * \mathrm{mmol}^{-1}]}$$

Equation 20

The reaction contained 2.3 mg/ml of immobilized hemoglobin. Reaction was started by addition of glucose to a concentration of 200 mM. The GOXCAT catalyst had an apparent activity of 0.45 U/ml and GOXHEMCAT 0.55 U/ml. After start of bubble aeration, +/- 20 µM of oxygen concentration were tolerated and corrected if they exceeded or fell below this values. Samples for gluconic acid concentration measurement were taken every half hour for 3 hours and one last sample taken after 22 h. For the oxygen concentration for catalyst with immobilized hemoglobin a high fluctuation of 30 µM and more per minute was observed. The oxygen concentration was controlled for 3 h and afterwards left at fixed flow.

Both catalysts reached oxygen saturating conditions after 10 h. Afterwards the reactor with hemoglobin showed a slightly higher oxygen concentration over a course of 4 hours (fig. 33). Both reactor runs achieved oxygen saturation of buffer after 10 hours.

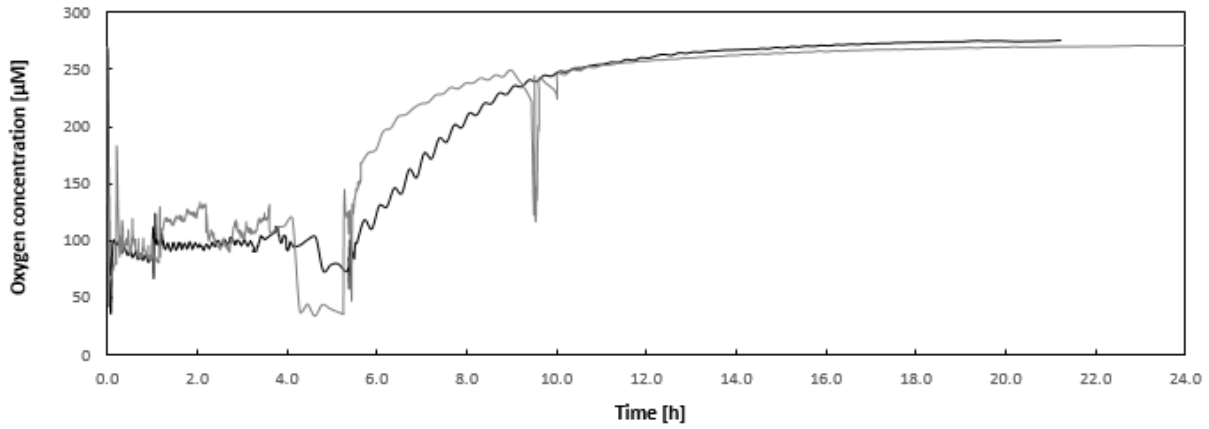

Figure 33*: Comparison of oxygen concentration fluctuation in a 50 ml reactor with immobilized GOX and catalase with and without hemoglobin at continuous bubble aeration, manually set to 100 µM. Oxygen depletion of the GOX and catalase co-immobilizate was 0.45 U/ml and 0.55 U/ml for the immobilizate with GOX, catalase and hemoglobin. The black line indicates the GOX catalyst with catalase and the gray line the GOX, hemoglobin and catalase catalyst.*

Enzyme activity started to decline after 6 h until oxygen supply was higher than consumption. After 6 h of reaction, the GOXHEMCAT was slightly better supplied with oxygen, but not as much to compromise the comparison of samples. The higher oxygen supply of the GOXHEMCAT catalyst was negligible and all gluconic acid samples could be evaluated.

Both reactor runs produced a very similar concentration of gluconic acid for at least 3 h (fig. 34). After 24 h an immense increase of product was measured in the reactor with immobilized hemoglobin, while the particles showed already visible degradation. Compared to previous experiments of surface aeration, the gluconic acid production during the first three hours showed still no clear benefit of hemoglobin on reaction.

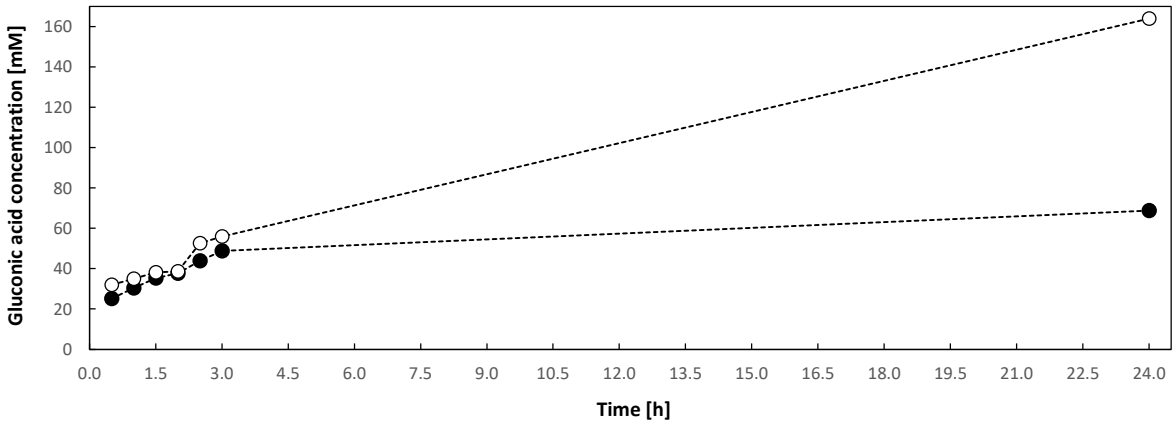

Figure 34: *Comparison of gluconic acid concentration over time of immobilized GOX and catalase with and without hemoglobin in bubble aeration reactor with 100 µM of oxygen concentration. Oxygen depletion of the GOX and catalase co-immobilizate was 0.45 U/ml and 0.55 U/ml for the immobilizate with GOX, catalase and hemoglobin. The GOX and catalase catalyst is shown as • and the GOX, hemoglobin catalase catalyst as ○.*

The particles of both reactor runs showed again strong mechanical abrasion over time, leading to release of GOX, hemoglobin and catalase into bulk. It was not clear if a release of hemoglobin by abrasion over time led to performance as dissolved hemoglobin, which always performed very efficient in previous experiments of surface aeration. In combination with the change of fixed oxygen concentration in bulk from saturating conditions to 100 µM of oxygen, the increase of gluconic acid concentration could be result of the implementation of hemoglobin.

The decrease of oxygen concentration to 50 µM in the next experiment should show how the course of gluconic acid was influenced by implementation of hemoglobin in a reaction with very oxygen limiting conditions.

3.4.2.3 Bubble Reactor with continuous oxygen concentration of 50 µM

The third reactor run was done at 50 µM of oxygen concentration. 170 U/g of GOX were offered to carrier and an apparent activity of 67 U/g was measured.

1.46 g of prepared particles were used for the reactor runs, which would theoretically lead to a concentration of 122.6 mM of GA (equ. 17).

$$122.6\ [mmol * h^{-1} * l^{-1}] = \frac{1.46\ [\mathrm{g}]\ *\ 67\ [\mu\mathrm{mol} * \mathrm{min}^{-1} * \mathrm{g}^{-1}] *\ 60\ [\mathrm{min} * \mathrm{h}^{-1}]}{0.05\ [\mathrm{l}] *\ 1000\ [\mu\mathrm{mol} * \mathrm{mmol}^{-1}]}$$

Equation 21

The oxygen depletion for immobilizate with GOX and catalase was 0.56 U/ml and 0.47 U/ml for immobilized GOX, hemoglobin and catalase. 2.3 mg/ml of hemoglobin were present in the reaction. Maintaining the control of bubble aeration, was more difficult than for previous reactor runs. High gas-flow fluctuations were observed during reactor run. The fluctuation range was set between 80 to 40 µM of oxygen but often exceeded or fell below this values.

The oxygen concentration of both catalysts stayed fixed at around 50 µM for 9 h and gradually increased after that. While the reactor run with GOXHEMCAT showed an oxygen saturation in bulk after 23 hours, the bulk of GOXCAT showed only a saturation of 200 µM (fig. 35).

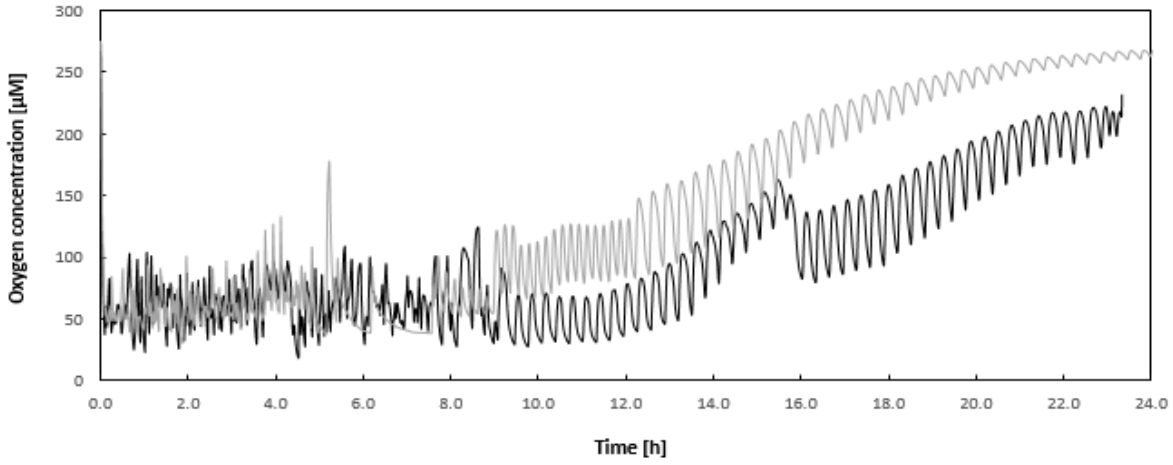

Figure 35: *Comparison of oxygen concentration fluctuation in a 50 ml reactor with immobilized GOX and catalase with and without hemoglobin at continuous bubble aeration, manually set to 100 µM. Oxygen depletion of the GOX and catalase co-immobilizate was 0.56 U/ml and 0.47 U/ml for the immobilizate with GOX, catalase and hemoglobin. The black line indicates the GOX catalyst with catalase and the gray line the GOX, hemoglobin and catalase catalyst.*

Both reactor runs showed a very similar oxygen concentration for at least 9 hours, which means that samples taken after this 9 h were not evaluated since a better performance could be the result of higher oxygen supply, not of hemoglobin. Comparing the oxygen saturation time point of both catalysts, the faster saturation of bulk for GOXHEMCAT indicated a faster loss of activity. The reactor runs with 50 mM of oxygen saturation however, showed a later oxygen saturation compared to previous reactor runs and therefore slower enzyme activity decrease. The loss of enzymatic activity seemed to correlate with oxygen flow intensity.

The comparison of gluconic acid showed an underperformance of GOXHEMCAT immobilizate compared to GOXCAT. The end concentration of both catalysts was very similar. After 24 h the GOXCAT catalyst showed a gluconic acid concentration of 41.8 mM while the GOXHEMCAT catalyst produced 40.1 mM (fig. 36).

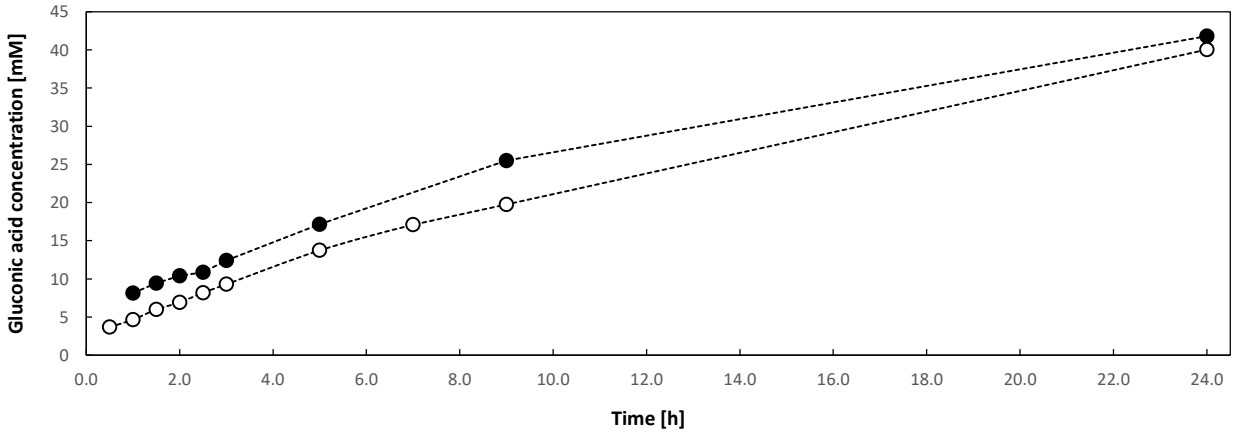

Figure 36: *Comparison of gluconic acid concentration over time of immobilized GOX and catalase with and without hemoglobin in bubble aeration reactor with 100 µM of oxygen concentration. Oxygen depletion of the GOX and catalase co-immobilizate was 0.56 U/ml and 0.47 U/ml for the immobilizate with GOX, catalase and hemoglobin. The GOX and catalase catalyst is shown as • and the GOX, hemoglobin catalase catalyst as ○.*

The reduction of oxygen concentration in bulk led to no positive effect on gluconic acid production by immobilized hemoglobin, but a slightly underperformance. Even the sample of 24 h, which was exposed to higher oxygen concentration, was lower than for the GOXCAT catalyst. When the reactor runs with 100 µM of oxygen concentration were compared, product concentration was drastically decreased by substrate limitation. While the substrate concentration was divided into half, gluconic acid concentration was only a quarter. At medium

GOX loadings of about 150 U/g substrate supply of GOX can´t only be enhanced by increasing mass-diffusion into porous carrier, but also by high oxygen supply in bulk.

A significant effect of hemoglobin couldn´t be determined at low oxygen concentration in bulk. The repeated underperformance of GOXHEMCAT indicated that hemoglobin is prone to disturbing factors and that causes likely an autoxidation reaction. The fact that bulk in reactor run with GOXHEMCAT had a shorter oxygen saturation time than the GOXCAT immobilizate supported this theory.

3.4.2.4 Conclusions

While hemoglobin and catalase (both immobilized) performed better in surface aeration reactions and caused a distinct gluconic acid increase compared to the GOXCAT catalyst, it was not as effective for bubble reactor runs. In the bubble aeration set-up the GOXHEMCAT often performed less efficient than the GOXCAT catalyst, except for the reactor run at 100 µM after 24 h of reaction. The change of set-up to bubble aeration seemed to be a possible trigger for autoxidation.

High oxygen input is a critical factor to trigger the spontaneous oxygen release and oxidation of hemoglobin, creating superoxide radicals that damaged GOX and led to a slight underperformance. Autoxidation by high oxygen supply is a known problem in science [44]. Literature research showed that hemoglobin is prone to autoxidation at high oxygen concentrations. The iron of heme group spontaneously oxidizes to inert methemoglobin by release of oxygen and by-production of superoxide radical $-O2^{\cdot -}$ (fig. 37). The superoxide radical induces further oxidation reactions.

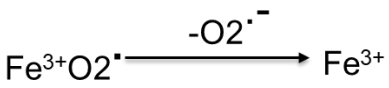

Figure 37: *Spontaneous autoxidation of oxyhemoglobin to methemoglobin by release of oxygen and by-production of superoxide radical.*

The rate of autoxidation of hemoglobin is much lower than for free heme groups. The embedding into globin structures has a protective effect against autoxidation according to F. B. Jensen from the Institute of Biology at the Odense University of Denmark [46]. The covalently linkage of hemoglobin during immobilization on Relizyme™ may have a negative impact on autoxidation probability of hemoglobin, by caused structure changes. The exposure of hemoglobin to oxygen in form of bubbles may start a radical chain reaction, leading to less activity of GOX. F.B. Jensen also states that oxygen bound by hemoglobin might be replaced by water molecule or OH^- to form aqua- or hydroxyl methemoglobin. The autoxidation correlates with a decreased amount of oxygen delivered by hemoglobin [47].

An impact on reaction by change of set-up as influencing factor couldn´t be excluded either, like a compromising of mechanical stability. During the reaction a strong mechanical degradation of particles bubble aeration could be observed. The immense mechanical abrasion of particles during bubble aeration was a severe drawback. The continuous reduction of mass-transport limitations by this abrasion made the differentiation between catalysts very difficult.

After a certain time of reaction enzymes could be present in dissolved form in reactor with fixed oxygen concentration, which led to non-mass-transport limiting and non-liquid limited conditions and made the implementation of hemoglobin obsolete, especially with peroxidase present to degrade accumulating peroxide.

The implementation of hemoglobin in set-up of bubble aeration led to by far less improved reactions than in reactor with surface aeration but allowed interesting insights in the behavior of hemoglobin.

4 Discussion and Conclusions

The characterization of hemoglobin confirmed its potential use as an oxygen vector. The spectrum analysis of oxygenated hemoglobin before and after the implementation in reduced form in a reaction with GOX showed a clear change of oxidation state of hemoglobin, either causing it to deoxygenate or oxidize to methemoglobin. The ability of oxygen release was studied and proven in unsaturated reaction buffer. Furthermore, hemoglobin was successfully tested for peroxidase activity by adjusting a spectrophotometric catalase assay. A low concentration of hemoglobin was able to degrade peroxide instantly in an enzyme-like fashion.

It was proven that hemoglobin could be used as an oxygen vector in reactions with dissolved and immobilized GOX. It was observed that it altered the initial reaction rate of non-aerated reactions by improving oxygen depletion in bulk and in porous carrier. Mass-transport limitations were determined as oxygen concentration gap between bulk and in particle by combination of use of oxygen sensor and sensor for luminescent intraparticle measurement. The combination of hemoglobin and catalase allowed to significantly reduce the mass-transport gap or even close it for loadings of 30 U/g and lower. Mass-transport limitations are, apart from flexibility loss and active side blocking of enzymes after reaction, the main reason for low efficiency and space time yield [27]. Hemoglobin showed the potential as an oxygen vector for immobilized enzymes by improving these mass-transport limitations into carrier and by oxygen supply in bulk.

Regarding the productivity, hemoglobin improved the product concentration by more than 3-fold when implemented under certain conditions. But the results also showed that hemoglobin is prone to disturbing factors. The enzyme-like ability to perform redox-reactions that allowed hemoglobin to work as a peroxidase was also the reason of its vulnerability to degradation and GOX damaging, causing underperformances in reaction.
The best results were achieved by implementation in surface aeration reactions with mild stirring conditions and non-fixed oxygen concentrations and co-immobilization of GOX and hemoglobin with catalase. The benefits of catalase could be identified and its benefit for implementation with hemoglobin confirmed.

The conditions of continuous bubble aeration in a batch reactor were suboptimal to achieve a production of high gluconic acid concentration, likely due to autoxidation susceptibility of hemoglobin in presence of high oxygen concentrations or mechanical abrasion of particles, causing mass-transport limitations of porous carrier to fade by release of GOX to bulk.

Concluded, the use of hemoglobin in oxygen consuming reactions is a potentially very effective and relatively simple method to improve the problem of oxygen limitation. It is sustainable, cheap, can be dissolved in high concentrations (100 mg/ml and more) and immobilized, which makes it useable for continuous processes. A screening for optimum conditions would be still necessary to prevent problems of autoxidation and poor performance. Reactions could benefit greatly if hemoglobin could be stabilized and could be used as a standardized oxygen vector in industrial processes.

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6 List of abbreviations

GOX	Glucose oxidase
Cat	Catalase
Hem	Hemoglobin
BSA	Bovine Serum albumin
CLEA	Cross-linked enzyme aggregates
Prefix i	immobilized
Prefix d	dissolved
HEMi	immobilized hemoglobin
HEMd	dissolved hemoglobin
CATi	immobilized catalase
CATd	dissolved catalase
U/g	Units per g/ µmol substrate converted per minute per g catalyst
Ruth dye	Tris (4,7-diphenyl-1,10-phenanthroline) ruthenium(II) dichloride
PBB	Potassium phosphate buffer
EtOH	Ethanol
Rpm	Rounds per minute
η	Effectiveness factor
OTR	Oxygen transfer rate
k_La	volumetric mass transfer coefficient

7 Appendix

Relizyme ™ HFA Ep403:

- Rigid metacrylic polymer matrix
- Average pore diameter 40-60 nm
- Particle size range 100-3001
- Characteristics:
  - High physical and chemical stability
  - Low swelling tendency in high molar solutions and in common solvents
  - High mechano-osmotic resistance gi [48]ven by intense cross-linking

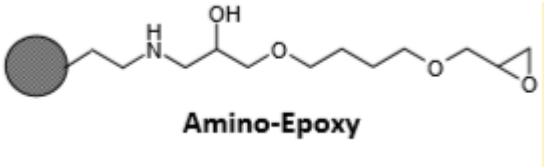

Figure 47: *Functional group of Relizyme™ HFA Ep403 particles.*

[48]

Glutaraldehyde:

- Cross-linking agent
- $C_5H_8O_2$
- Aldehyde
- Precautions for working:
  - Severely irritating to skin, eyes and respiratory system
  - Causes headache, nosebleed, chest tightness, nausea, vomiting, dermatitits and asthma
  - Use of gloves
  - Work under exhaust hood

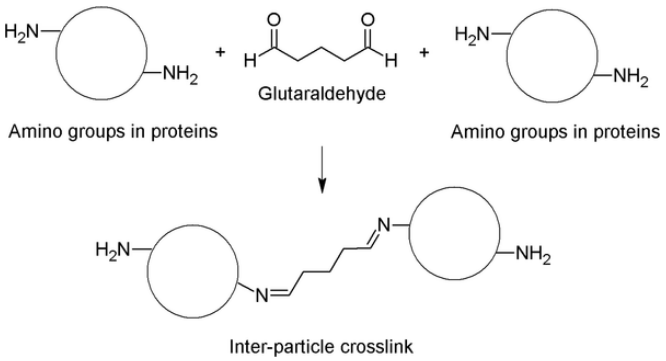

Figure 48: *Production of CLEAs by cross-linking of enzyme with glutaraldehyde.*

[49], [50].

Oxygen Depletion Measurements:

Table 13: *Characterization of cross-linking of 0.2 mg/ml GOX with BSA in different ratios.*

BSA [mg/ml]	0	2.0	4.0
Stock [U/ml]	83.5		
Supernatant [U/ml]	25.7	42	32
Pellet re-diss. [U/ml]	9.0	7.4	5.9
Pellet percentage [%]	15	13	10
Yield [%]	69	50	62
CLEAs [U/ml]	4.5	21.9	20.4
CLEAs percentage [%]	5	26	24
η [%]	8	53	40

Table 14: *CLEAs with GOX in different concentrations and additive in tenfold ratio.*

	BSA			Hemoglobin		
Additive [mg/ml]	10	20	40	10	20	40
GOX [mg/ml]	1.0	2.0	4.0	1.0	2.0	4.0
Stock U/ml]	1,434	4,619	12,252	-	-	-
Ox. depletion [U/ml]	-	-	-	125	206	883
Supernatant [U/ml]	134.9	345.4	621.7	10.4	16.9	27.7
Pellet [U/ml]	4.1	9.9	15.3	0.2	0.5	1.0
Yield [%]	90.6	92.5	94.9	-	-	-
CLEA [U/ml]	22	51	144	1.0	5.1	14
η [%]	-	-	-	1.7	1.2	1.2

Table 15: *Immobilization of low GOX loadings and hemoglobin on Relizyme™.*

Loading [U/g]	2	3.5	7.5	21	38	70
Lost activity [U/ml]	0.13	0.2	0.3	1.3	2.6	4.4
Yield [%]	35.4	57.1	55.5	40.5	31.5	36.2
GOX mono-imm.[U/g]	1.16	2.0	8.6	17.8	28.0	32.6
GOXHEMi [U/g]	0.1	3.0	4.4	7.4	9.0	12.1
GOXHEMd [U/g]	0.01	5.9	13.0	25.2	27.1	30.9
η GOX mono [%]	166	102	207	209	237	131

Table 16: *Immobilization of medium high GOX loadings and hemoglobin on Relizyme™ and ox. Depletion measurement of dissolved catalase.*

Loading [U/g]	80	150	350
Lost activity [U/ml]	0	2.6	8.7
Yield [%]	100	82	74
GOX mono-imm [U/g]	24	46	149
GOXCATd [U/g]	62	15	81
GOXHEMi [U/g]	10	151	61
GOXHEMiCATd [U/g]	16.5	35	79
GOXHEMd [U/g]	38	61	173
GPXHEMdCATd [U/g]	21	59	52
η GOX-mono [%]	31	39	60

Table 17: *Immobilization of medium high GOX loading, hemoglobin and catalase on Relizyme™.*

Loading [U/g]	200
Lost activity [U/ml]	12.4
Yield [%]	40.1
GOX mono-imm. [U/g]	83
GOXCAT [U/g]	45
GOXHEMi [U/g]	67
GOXHEMiCati [U/g]	91
GOXHEMd [U/g]	76
GOXHEMdCati [U/g]	51
η GOX mono [%]	101

Table 18: *Immobilization of high GOX loadings and hemoglobin.*

Loading [U/g]	284	424	730	1087	1583	2400
Lost activity [U/ml]	16.2	32.2	32.4	73.8	100.6	114.5
Yield [%]	43	26	56	32	36	52
GOX mono-imm [U/g]	239	280	266	161	304	169
GOXHEMi [U/g]	218	160	331	333	248	398
GOXHEMd [U/g]	108	160	141	201	440	161
η GOX-mono [%]	195	251	66	45	53	13

Gluconic acid concentration measurements:

Table 19: *Gluconic acid measurement of low GOX loadings and hemoglobin in reaction with 25 mg/ml of carrier.*

Loading [U/g]	2	3.5	7.5	21	38	69
GOX mono-imm. [mM]	8	12	22	34	48	52
Calc. from IRR [mM]	16	28	118	244	384	448
Ratio [%]	49	44	19	14	13	12
GOXHEMi [mM]	20	36	53	78	69	102
Calc. from IRR [mM]	2	41	61	102	124	166
Ratio [%]	126	130	44	32	18	23
GOXHEMd [mM]	25	42	89	117	106	122
Calc. from IRR [mM]	0	81	178	346	372	423
Ratio [%]	159	152	75	48	28	27

Table 20: *Gluconic acid measurement of high GOX loadings and hemoglobin in reaction with 1.25 mg/ml of carrier.*

Loading [U/g]	284	424	730	1087	1583	2400
GOX mono-imm. [mM]	10	13	23	21	27	34
Calc. from IRR [mM]	239	280	266	161	304	169
Ratio [%]	4	5	9	13	9	20
GOXHemi [mM]	7	12	12	14	15	16
Calc. from IRR [mM]	218	160	331	333	248	398
Ratio [%]	3	7	4	4	6	4
GOXHEMd [mM]	48	45	49	55	59	65
Calc. from IRR [mM]	108	160	141	201	440	161
Ratio [%]	44	28	34	27	13	40

Table 21: *Gluconic acid measurement of medium high GOX loadings, hemoglobin and dissolved catalase in reaction with 25mg/ml of carrier.*

Loading [U/g]	80	150	350
GOX mono-imm. [mM]	8	11	13
Calc. from IRR [mM]	105	196	640
Ratio [%]	8	5.6	2.1
GOXCATd [mM]	25	26	30
Calc. from IRR [mM]	265	65	346
Ratio [%]	23.8	13.5	4.8
GOXHEMi [mM]	6	8	14
Calc. from IRR [mM]	41	121	648
Ratio [%]	5.6	4.3	2.1
GOXHEMiCatd [mM]	10	13	27
Calc. from IRR [mM]	71	152	340
Ratio [%]	9.6	6.8	4.2
GOXHEMd [mM]	17	19	27
Calc. from IRR [mM]	163	262	740
Ratio [%]	16.6	9.8	4.3
GOXHEMdCAtd [mM]	26	29	40
Calc. from IRR [mM]	89	253	224
Ratio [%]	24.4	15.1	6.2

Table 22: *Gluconic acid measurement of 200 U/g of GOX with hemoglobin and immobilized catalase in reaction with 2.5 mg/ml of carrier over a time course of 6 h.*

Time [h]	1	2	3	4	5	6
GOX mono-imm [mM]	4	7	8	9	10	11
Calc. from IRR [mM]	12	24	37	49	61	73
Ratio [%]	32	27	22	19	17	15
GOXCati [mM]	-	5	7	8	10	10
Calc. from IRR [mM]	7	13	20	26	33	40
Ratio [%]	-	22	20	17	16	14
GOXHEMi [mM]	5	8	11	12	15	16
Calc. form IRR [mM]	10	20	29	39	49	59
Ratio [%]	38	34	30	26	24	22
GOXHEMiCati [mM]	11	18	22	25	29	31
Calc. from IRR [mM]	13	27	40	53	66	80
Ratio [%]	89	72	61	51	48	42
GOXHEMd [mM]	15	17	26	27	26	33
Calc. from IRR [mM]	11	22	32	43	54	65
Ratio [%]	119	72	70	55	42	45
GOXHEMdCati [mM]	16	26	34	35	40	32
Calc. form IRR [mM]	7	22	32	43	54	65
Ratio [%]	134	107	94	71	66	43