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Functionalized porous ceramic microbeads as carriers in enzymatic tandem systems

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ABSTRACT

Porous Al_2O_3/SiO_2 microbeads (MBs) are propped as carriers for enzymes used in flow reactor setups for tandem enzymatic reactions. Glucose oxidase (GOx) and catalase (CAT) perform sequential reactions. GOx catalyzes the conversion of glucose and O_2 to gluconic acid and H_2O_2. CAT decomposes H_2O_2 to H_2O and O_2. Spherical MBs were fabricated via ionotropic gelation followed by rapid-sintering (1200 °C/5 min), featuring reasonably high open porosity (~50%), appropriate specific surface area ($30 \pm 5\ m^2/g$) and multi-modal pore sizes ($d_{50} = 79$ nm) for envisaged enzyme immobilizations. After activation and amino-silanization, GOx and CAT were successfully immobilized onto MBs applying an EDC/sulfo-NHS crosslinking reaction. Under varied pH and temperature conditions, the immobilized enzymes showed advantages towards harsh conditions (pH 2; 70 °C) and demonstrated high activities at neutral pH and 21 ± 2 °C (RT), serving as optimal conditions for flow experiments. By varying flow rates (0.1–6 ml/min) in individual reactor compartments, GOx- and CAT-functionalized MBs achieved satisfiable H_2O_2 conversion rates at 1.0 ml/min for 24 h. In tandem use, obtained H_2O_2 is constantly degraded over time, maintaining high enzymatic overall performances for two more operation cycles. The presented strategy is particularly interesting, as it can potentially be transferred to other multi-enzyme reaction systems.

1. Introduction

In the field of biochemical engineering and biotechnology there is no such thing as 'the universal' support material for enzyme immobilization. Especially in vitro multi-enzymatic approaches [1,2], which are highly complex processes, benefit from numerous existing support materials and immobilization techniques to select from.

We would like to introduce and provide new perspectives to the industrial sector [3] by examining the possibility and efficiency of single porous ceramic MBs with adjustable surface and pore characteristics, composed of Al_2O_3/SiO_2 as carrier material for two enzymes, namely glucose oxidase (GOx, EC number = 1.1.3.4) and catalase (CAT, EC number = 1.11.1.6). More particularly, we further evaluate the feasibility for an enzymatic tandem reactor system, realizing controlled turn-over rates, by placing the functionalized MBs with GOx and MBs with CAT in a sequential order while analyzing the substrate conversion.

In particular, available supports can be divided into (1) organic materials and (2) inorganic materials such as natural SiO_2 (diatoms), zeolites, metals and non-metallic ceramic oxides, which are already successfully used as enzyme support materials [3,4]. Overall, regarding enzyme immobilization ceramic nanostructured carriers such as nanoparticles, nanofibres, micro- and nanotubes [5], as well as nanoporous membranes are preferably used [6], the latter makes up a significant proportion in the area of enzyme-based membrane reactors. Precisely, the surface curvature of the support as well as the pore size should be optimized with respect to the enzyme dimensions (nm range) to be successfully physioadsorbed as compiled by Sang and co-workers [7,8]. For example, lysozyme functionalized ZrO_2 microtubes (pore sizes ≤ 0.2 μm) showed highly promising results for bacteria filtration [9], other Al_2O_3 substrates (pores size 5–300 nm) were ideal candidates for characterizing enzyme layer formation, stability and reusability potential [10,11]. In turn, ceramic foams (average pore sizes of 45 μm and 77 nm) therefore benefit from low internal mass transfer resistance which enhances the enzymatic turn-over rates, when compared to nanoporous materials [12]. Generally, open foams

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lack nanostructured porosity thus the pore size is greater than the size of the enzyme. To overcome that aspect a covalent immobilization strategy is favored, e.g. mediated by aminosilanization, as suggested by other working groups [13,14]. Regarding to our studies it has already been shown that 3D-assembled ceramic MBs (15 nm intraparticle pores and 105 μm interbead pores) were used efficiently as carrier system for the enzyme laccase [15].

Considering all these given limitations and possibilities, it is not only desired to develop and apply increasing numbers of carrier materials, but also to investigate given materials in more complex correlations by multiple enzymatic approaches.

In this regard the invention to apply MBs as carrier materials especially in enzyme functionalization based processes meets several existing needs: (1) It's a simple, rather eco-friendly processing route, optimal for rapid production of high industrial amounts, as developed in the previous work from Klein et al. [15,16] and enhanced by Mullens and colleagues [17]; (2) Furthermore, the material shows a high accessible surface area (30 ± 5 m^2/g), (3) a nano-scaled intra-pore-system ($d_{50} = 79$ nm) suitable for stable enzyme binding and a high flow permeability due to the interpore spaces between single MBs (10-100 μm) as required for an ideal carrier material. (4) Ceramic MBs show a main advantage over other organic support materials, such as natural (e.g. alginate, collagen, cellulose and starch) and synthetic polymers (e.g. polystyrene, polyacrylamide, polyamide), in terms of their thermostability, solvent stability, mechanical benefits and certainly their reusability, even if they are more costly in the beginning. (5) Furthermore assembled MB beds can be created in desired dimensions and various mixtures.

GOx and CAT have been chosen, as they are widely used biocatalysts, particularly as model enzymes for tandem reactions since several decades [7,18–22]. Both GOx and CAT have already found application as enzymatic tandem system in food processing (e.g. removal of glucose from egg-white, antioxidative enzyme systems to remove active oxygen species, notably H_2O_2), in propulsion driven nanomotor studies, in analytical practice (e.g. hypoxia in cell culture), in medical devices (e.g. bio-sensors) and e.g. as membraneless glucose biofuel cells 'GBFCs' as power generators [19,23–26]. A particular advantage is, that the reaction products of those enzymes are easily detectable in situ, which can give direct information about the ongoing performance of the functionalized support.

GOx catalysis the oxidation of ß-glucose to glucono-1,5-lactone which spontaneously hydrolyses non-enzymatically to gluconic acid using molecular oxygen (O_2) as co-factor and releasing hydrogen peroxide (H_2O_2). The enzyme CAT converts H_2O_2 to H_2O and O_2. As represented by the reaction equations EI and EII, GOx and CAT show a self-enforced effect and can be used together when net H_2O_2 production has to be avoided. In this study the enzyme activity for both enzymes is defined as "1 Unit (U) = 1 μmol H_2O_2/min" and as specific activity in "Units per gram material = U/g MB".

$$(EI) O_2 + H_2O_2 + C_6H_{12}O_6 \rightarrow \text{conversion by GOx} \rightarrow C_6H_{12}O_7 + H_2O_2$$

$$(EII) 2H_2O_2 \rightarrow \text{conversion by CAT} \rightarrow 2H_2O + O_2$$

The immobilization of GOx and CAT has been described for example in TiO_2 and Al_2O_3 carriers ($d_{50} = 17.5$–85.5 nm; specific surface area = 9–100 m^2/g) [7], for MgO_3Si-based inorganic carriers (Florisil®, mesh size = 60-100; specific surface area = 170–300 m^2/g), in polyacrylonitrile membranes (pore size 10–200 nm; specific surface area = 10-30 m^2/g) and in multiwall carbon nanotubes (MWCNT) used as catalytic GBFCs [7,19,20,26].

In that regard, configuring an enzymatic tandem system using the versatile characteristics of ceramic MBs, in combination with two known enzymes, which are frequently investigated in terms of immobilization and practicable in real applications as such, will provide new opportunities in the industrial sector.

In this study, the average pore size is around 5 times higher than the size of GOx ($7.5 \times 6.2 \times 3.8$ nm^3) and CAT ($10.7 \times 9.4 \times 7.4$ nm^3) (supplementary work), therefore a covalent immobilization strategy is favored, as suggested by other working groups [13,14].

A variety of parameters were studied and compared systematically: (1) separated single batch experiments were performed to determine the optimal activity conditions (i.e. pH variation and temperature differences) of the covalently immobilized enzymes compared to the free enzymes. Based on the pre-determined conditions, (2) the enzyme-functionalized MBs were transferred to a tandem reaction setup in laboratory scale, observing the overall performance of the system by analyzing the decomposition of glucose (EI) and the production of H_2O_2 (EII) under different flow rates and the persistence towards repeating reaction cycles.

2. Materials and methods

2.1. Materials

For the fabrication of ceramic MBs α-Al_2O_3 colloidal particles ($d_{50} = 205$ nm, Taimei Taimicron, TM-DAR, Krahn Chemie, Germany) and SiO_2 sol ($d_{50} = 7$ nm, BINDZIL® 30/220, NH_3-stabilized, Akzo Nobel, Germany) were used. Sodium alginate (product number A3249) and tri-sodium-citrate dihydrate (product number A0548) were purchased from AppliChem (Germany). The cross-linking solution was prepared with calcium chloride dihydrate (product number 21102, Fluka, Germany) and absolute ethanol (AnalaR, 20821-321, VWR, Germany). For surface functionalization experiments 3-aminopropyltriethoxysilane (APTES, 99%, product number 440140), anhydrous toluene (99.8%,), orange II sodium salt (product number 75370), hydrochloric acid solution (HCl, product number 35328) and sodium hydroxide (NaOH, product number 71692) were purchased from Sigma-Aldrich, Germany. For enzyme immobilization glucose oxidase (GOx) from *Aspergillus niger* (EC number = 1.1.3.4, $M_W = 186.000$ Da, > 15000 U/mg, product number G6125-10KU, 1 U = 1 μmol H_2O_2/min at pH 5, 35 °C) and catalase (CAT) from bovine liver (EC number = 1.11.1.6, $M_W = 250.000$ Da, 2000–5000 U/mg, product number C9322, 1 U = 1 μmol H_2O_2/min at pH 7, RT) were purchased from Sigma-Aldrich (Germany) and covalently immobilized using N-hydroxysuccinimide (NHS, 97%, product number 56480) and N-Ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 98%, product number E6383) obtained from Fluka (Germany) and Sigma-Aldrich (Germany), respectively. For enzymatic activity tests D-(+)-glucose (product number G8270, Fluka, Germany) and hydrogen peroxide (30% w/w, product number 31642, Sigma-Aldrich, Germany) were used as substrates. A 50 mM K_2HPO_4 buffer was freshly prepared and used for all enzyme experiments (product number 04248, Riedel-de-Häen, Germany). For all experiments double deionized water (ddH_2O) (conductivity of 0.04 μS cm^{-1}, Merck Millipore, Germany) was used.

2.2. Fabrication of ceramic microbeads

Porous ceramic MBs were prepared by an ionotropic gelation route as described by Klein et al. [16]. The processing route is divided into four main parts: (1) suspension preparation, (2) bead formation, (3) drying and (4) sintering of the obtained green bodies. Briefly, an aqueous ceramic suspension was prepared by homogeneously mixing SiO_2-Sol as colloidal suspension (66.5 g), sodium citrate as dispersant (0.4 g), sodium alginate as gelling agent (1.6 g) and Al_2O_3 nanoparticles as ceramic matrix (39.9 g) in water. Subsequently, the suspension was added dropwise into a cross-

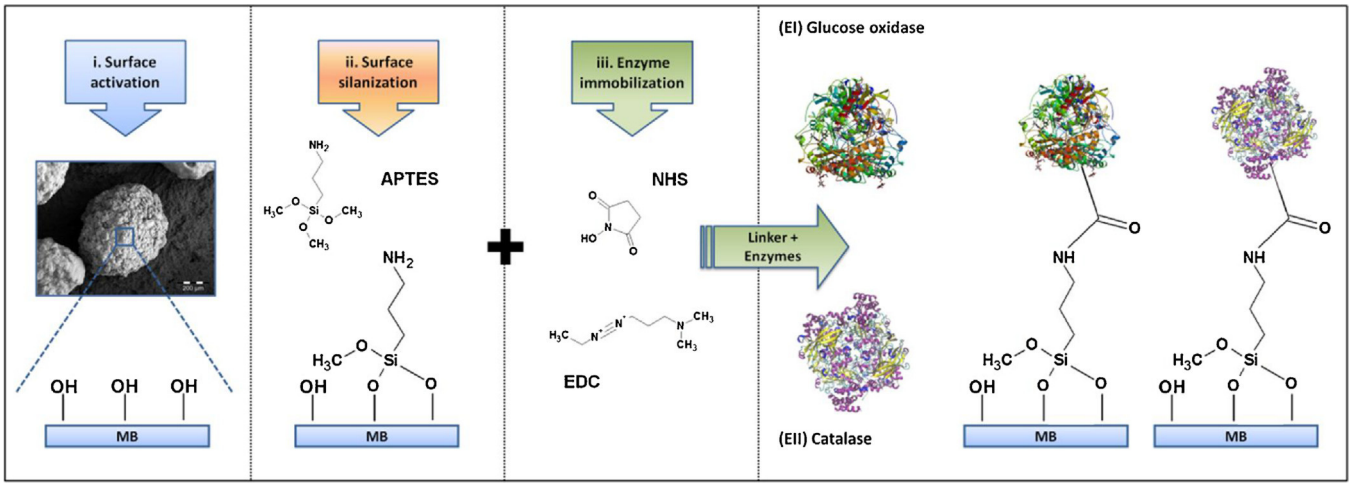

Fig. 1. Surface functionalization and enzyme immobilization of ceramic microbeads (MB). The MBs surface is (i) hydroxyl-activated followed by (ii) functionalization with amino-silane precursors (APTES) and (iii) covalent immobilization of (EI) glucose oxidase (GOx) and (EII) catalase (CAT) adding the linker molecules EDC and NHS.

linking solution ($CaCl_2$, 0.1 mol/L) using a trimmed injection needle (Ø = 0.55 mm, sterican, Braun, Germany), instantly forming spherical MBs due to the formation of insoluble alginate/Ca^{2+} meshworks incoperating the Al_2O_3 and SiO_2 nanoparticles. Afterwards, prepared MBs were collected and washed with water, directly freeze dried (P8K-E-80-4, Piatkowski, Germany) and rapid sintered in a tube furnace at 1200 °C for 5 min (VTF1, Vecstar, United Kingdom) to burnout the organic material and create mechanically stable open-porous MBs [27].

2.3. Characterization of ceramic microbeads

The morphology and size of the MBs was examined by scanning electron microscopy (SEM, Field-emission SUPRA 40, Zeiss) and by using a two-point sizing approach (n = 200) in digital images (Keyence, VHX 500F, Germany). The pore size distribution, average pore diameter (d_{50}) and open porosity were analyzed by mercury-intrusion porosimetry (Pascal 140/440, POROTEC, Germany). The specific surface area (S_{BET}) was calculated from nitrogen adsorption isotherms recorded at −196 °C (Belsorp-Mini, Bel Japan, Japan) according to the BET-method. Before measurements, all samples were gassed out over night at 120 °C using argon. Helium-pycnometry (AccuPyc 1330, Micomeritics, Germany) was applied to measure the density and open porosity. To analyze the ζ-potential at the solid/liquid interface of the MBs, streaming potential measurements were performed using an electrokinetic analyzer for solid samples (SurPASS, Anton Paar, Germany). The solvent stability and dissolution behavior of sintered Al_2O_3-SiO_2 MBs were shown by analyzing the loss of calcium-ions (FLUITEST® CA CPC CALCIUM, Analyticon, Germany) from the porous MBs, when 0.5 g MBs were incubated for 7 days at RT under constant gentle shaking in 10 ml solvent solution, either water, HCl (1.0 M, pH 3), NaOH (1.0 M, pH 14) or ethanol [27].

2.4. Surface functionalization and enzyme immobilization

Fig. 1 summarizes the functionalization strategy involving surface activation by hydroxylation (i), surface silanization using APTES (ii) and finally covalent enzyme immobilization (iii) of GOx and CAT, respectively. Representation of the secondary structure and estimating the dimensions of GOx and CAT was done using the software Visual Molecular Dynamics (VMD, Version 1.9.1) as shown in the supplementary part. The atomic coordinates were taken from the RCSB Protein Database (PDB entry GOx #1GPE and CAT #1TGU).

Amino-silanization of sintered MBs using APTES was investigated in terms of different hydroxylation procedures for activation (acidic and hydrothermal treatment), different solvents (anhydrous toluene versus 90 vol.% ethanol/water mix) and different silanization times (0.5, 1, 2, 4, 8, 16 and 20 h), to maximize the accessible NH_2-groups on the surface for further immobilizing GOx and CAT. MBs were activated by acidic hydroxylation, covering 10 g MBs with freshly prepared Piranha solution (97% H_2SO_4: 35% H_2O_2, 3:1 (v/v)) for 30 min at RT [28] or by 20 min hydrothermal treatment using an autoclave at 120 °C and 2 bar (Systec 2540, Germany) [29]. MBs were then washed with water and finally dried at 70 °C for at least 3 h.

Activated MBs were subsequently amino-functionalized by either (i) refluxing 10 g of the material in 100 ml anhydrous toluene containing 2 vol-% APTES precursor at 85 °C under constant stirring (adapted from [30]), or otherwise (ii) by immersing 0.2 g activated MBs in 1.5 ml of 90 vol-% ethanol mixture containing 2 vol-% APTES under continuous shaking at 1000 rpm on a thermoshaker (ThermoMixer C, Eppendorf) at 75 °C. Afterwards, the MBs were washed with either toluene or ethanol and water followed by final drying at 70 °C over night. In both procedures (i) and (ii) the silane precursor was added when the basic reaction solution reached the silanization temperature. All experiments were performed in triplicates.

The number of accessible NH_2-groups on the MB surface was determined by using photometrical acid orange II (AO II) assay at λ = 483 nm [31]. To identify unspecific binding of AO II molecules hydroxyl-activated MBs served as reference (Fig. 1i.).

To achieve high specific enzyme activities, GOx and CAT were covalently immobilized using the linker molecules EDC (2 mM) and NHS (5 mM) related to Halfer et al. [32]. Thus, 1 g amino-functionalized MBs were dispersed in 5 ml phosphate buffered solution (pH 7, 50 mM) containing the linker molecules and GOx (0.5 mg/ml) or rather CAT (0.5 mg/ml). MBs were placed in a shaker (100 rpm) for 20 h at 4 °C. After enzyme immobilization, the MBs were washed with phosphate buffer and finally stored at 4 °C until use.

2.5. Catalytic activity assay by monitoring H_2O_2 contents: single batch experiments

The activity of immobilized GOx and CAT was analyzed systematically regarding their optimal reaction conditions in single batch experiments as represented in Fig. 2 before their application in the tandem reactor system. Thus the kinetic reaction rates were ana-

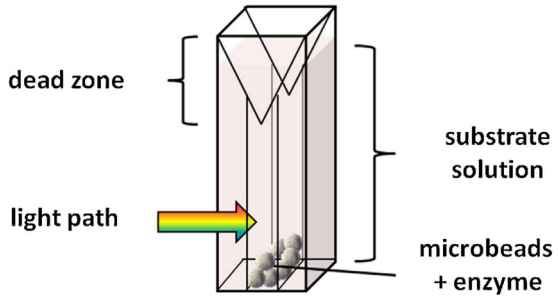

Fig. 2. Experimental setup for the single batch enzyme activity tests using a standard UV-cuvette containing the substrate solution and the catalysts (MBs immobilized with enzyme or free enzyme in solution).

lyzed by monitoring (EI or EII) the time-dependent increase (GOx) or decrease (CAT) of the absorbance at λ = 240 nm. This absorbance was related to the H_2O_2 concentration in solution by using calibration curves of different H_2O_2 concentrations (3–30 mM) in phosphate buffer (pH 7) and in D-(+)-glucose buffered solution (100 mM) (Fig. S1, correlation coefficient = 1.0 and 0.99, respectively). In standard experimental procedures, 0.15 g MBs (dry weight) were added to a standard UV-cuvette as described in Fig. 2. The reaction was started by adding 2 ml substrate solution to the cuvette (for GOx: 100 mM D-(+) glucose; for CAT: 32 mM H_2O_2). The assay was carried out by gently inverting the cuvette during defined intervals (GOx: 1 min; CAT: 30 s) over the whole assay time (up to 60 min), ensuring a sufficient contact of substrate and immobilized enzymes. The activity of the free enzymes was measured with free GOx (0.1 mg/ml) and free CAT (0.005 mg/ml) using the above mentioned substrate solution concentrations and treated under same conditions. The enzyme activity is reported as (i) the absolute change of absorbance over time (OD λ = 240 nm), resulting in (ii) the activity of the enzymes described as the velocity turnover (dP/dt, GOx $_{V30min}$, CAT $_{V5min}$) in μmol H_2O_2/min, 1 Unit (U) = μmol H_2O_2/min. By this, the specific enzyme activity for (iii) the free enzymes as U/mg of enzyme or for (iv) the immobilized enzymes in U/g of MBs material can be calculated. All experiments were performed in triplicate.

2.5.1. Effect of pH and temperature on the enzyme activity

Each enzyme works best within a defined range of pH and temperature. For GOx a pH of 5.5 with a broad range of activity (pH 4–7) and a temperature below 50 °C are stated as optima [33,34]. For CAT a neutral pH of 7 and ambient temperature of 37 °C is given as optimal conditions, highly depending on the enzyme specie [35]. The enzyme activities were determined exactly as previously described (Section 2.5). The pH stability was tested by changing the pH of the substrate solution (pH 2–10), while keeping the temperature constant at 21 ± 2 °C (RT) during the activity measurements. The thermal stability was analyzed by incubating the enzyme-immobilized MBs, as well as the free enzymes for 24 h in standard phosphate buffer (pH 7) at different temperatures (4 °C, RT, 50 °C and 70 °C). By analyzing the activity of the enzymes after they are exposed to such stresses, fundamental information about the enzyme activity and their limitations of application after immobilization compared to the free state could be addressed.

2.6. Flow reactor setup

In contrast to the single batch experiments using single MBs in cuvettes, an easy to use laboratory scaled continuous flow reactor was designed for analyzing the enzymatic tandem system (Fig. 3). It is composed of two subsequent compartments each containing 1.2 g functionalized MBs (EI = GOx and EII = CAT), separated by a small layer of cotton. The reaction vessel was filled with 30 ml phosphate buffered substrate solution (pH 7) containing 100 mM glucose or 32 mM H_2O_2 and circulated by a peristaltic pump under a defined flow rate at RT. By taking samples at defined intervals (1 or rather 2 h) the changes in the solution concentration in the reaction vessel were monitored. For H_2O_2 determination 2 ml samples were photometrically measured at 240 nm and returned to the reaction solution. For determination of the glucose concentration 100 μl samples were analyzed using the *ACCU CHEK* Aviva enzyme based sugar measuring device (Roche Diabetes Care Deutschland GmbH). Fig. S2 presents the calibration curve.

2.6.1. Stability and activity of immobilized GOx and CAT under different flow rates

It is of high importance to investigate the stability of the immobilized enzymes over time and under different flow conditions, in particular, for finding an useful flow rate to analyze both enzyme reactions (EI and EII) sequentially. A pH 7 buffered solution (no substrate, RT) was flushed through the reactor under different flow rates (0.1, 1.0 and 6.0 ml/min) using only GOx- or CAT-functionalized MB compartments (1.2 g of MBs). Before each activity measurement air was pumped through the system to get rid of excessive buffer and eventually weakly bound enzymes. Afterwards, the reactor was flushed with substrate solution (pH 7, RT) for 10 min using glucose or H_2O_2 at the same flow rate (0.1, 1.0 and 6.0 ml/min). The outflow was collected for photometrical analysis at 240 nm. The enzyme activity is given as consumption or production of percental H_2O_2 (μmol/min) and as specific activity (U/g MB).

2.6.2. The reactor setup in tandem use

Both compartments (EI and EII) of the tandem reactor system were filled with 1.2 g of GOx- and CAT-immobilized MBs in sequential order. The reactor was flushed with 30 ml glucose substrate solution (100 mM, pH 7, RT) at a flow rate of 1.0 ml/min in circulation mode. The concentration of H_2O_2 and glucose in the solution was monitored for 24 h, using intervals of 1–2 h. H_2O_2 was measured photometrically at 240 nm (samples were returned to the solution) and the glucose concentration was analyzed using 100 μl samples in an enzyme based sugar measuring device (*ACCU CHEK Aviva*). The enhancement of the operational stability of the immobilized enzymes was investigated by three consecutive 24 h cycles. When the first reaction cycle was finished the system was cleaned by rinsing with phosphate buffer (pH 7, RT) and fresh substrate was added to start the catalytic reaction as before.

For comparison, following references were tested: (i) 1.2 g MB only immobilized with GOx, (ii) free GOx (0.05 mg/ml) and (iii) a combination of free GOx (0.05 mg/ml) and free CAT (0.005 mg/ml) in tandem. For the operational stability of the free enzyme references (ii and iii), the free enzymes were added directly to the substrate solution and were rinsed through the system as previously described, certainly without MBs in the reactor. After 24 h and 48 h fresh glucose substrate (final concentration of 100 mM glucose) was added to the starting solution containing the enzymes.

3. Results and discussion

3.1. Properties of ceramic microbeads

Fig. 4A presents SEM micrographs of fabricated Al_2O_3-SiO_2 MBs and their surface characteristics showing a spherical bead morphology and a rather smooth surface. Suitable properties for enzyme immobilization were realized by sintering the MBs rapidly for 5 min at 1200 °C, showing a pore size distribution with inter-pores of ~10–1000 nm (d_{50} = 79 nm) and intra-bead pores of ~5–100 μm, a high open porosity of 56% and relatively high specific surface area of 30 ± 5 m^2/g were produced (Fig. 4B). What attracts attention,

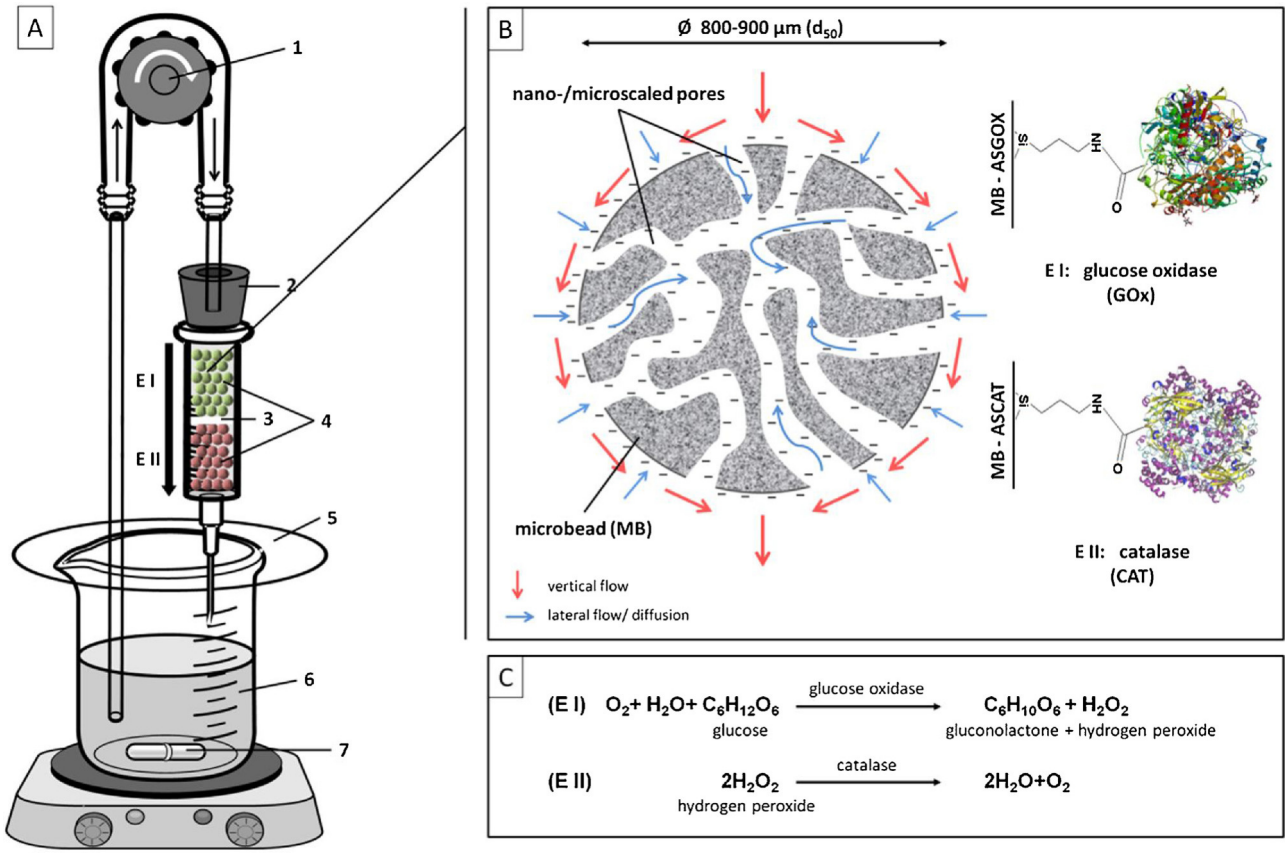

Fig. 3. Illustration of the tandem-flow reactor set-up filled with enzyme-functionalized MBs. (A) 1 = peristaltic pump; 2 = rubber plug; 3 = separation layer; 4 = MBs with immobilized enzymes; 5 = cover; 6 = substrate solution; 7 = stirring bar. (B) Enlarged depiction of a single porous MB showing its potential flow condition and the chemical enzyme bonding. (C) Enzymatic catalyzed reaction of glucose oxidase (GOx, EI) and catalase (CAT, EII) using glucose as initial substrate.

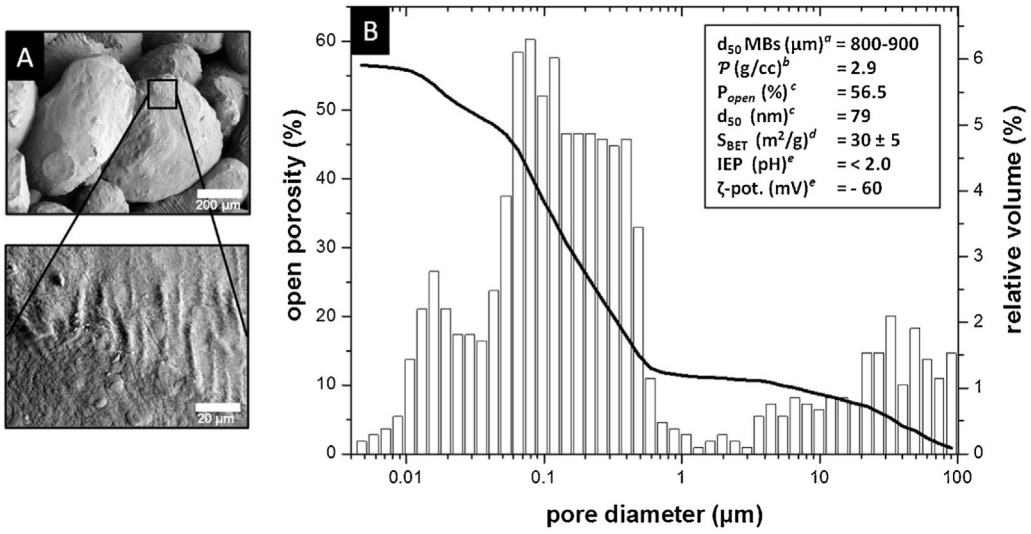

Fig. 4. Properties of sintered Al_2O_3-SiO_2 MBs (1200 °C for 5 min). (A) SEM images of single MBs and their surface characteristics. (B) MB size distribution (d_{50} MBs), material density (*P*), pore size distribution (graph), open porosity (P_{open}), inter-bead pore size (d_{50}) specific surface area (S_{BET}), the isoelctric point (IEP) and the surface potential (ζ-pot.) were obtained by [a] optical microscopy; [b] He-pycnometry; [c] Hg-porosimetry; [d] N_2-adsorption and [e] streaming-potential measurements.

when comparing with literature values [16] is the large average bead size of d_{50} = 800-900 µm and the wide size distribution of d_{10} = 500 µm to d_{90} = 1100 µm (Fig. S3) after sintering. This outcome certainly results from manual fabrication using a syringe, unlike using a controlled vibrating nozzle preparation demonstrating a more homogenous bead size distribution suggested by Roosen et al. [17]. With increasing sintering temperature (800–1200 °C) an increased chemical stability of the MBs towards acidic and basic solvents was demonstrated as expected (Fig. S4). This confirms the applicability of the material in a broad range of fluids. The interpores (~10100 µm) between the MBs, which are attributed to 10% of the open porosity (Fig. 4B), could potentially enable great liquid throughput, as for the transportation of substrates and products, with small pressure losses as previously decribed by Klein et al.

[15]. Because of the negative surface charge (-60 mV) of the material (IEP at pH 2) the lack of electrostatic interaction with the negativly charged enzymes under neutral pH conditions, as well as the relatively large pores the MBs were functionalized with amino-groups to provide sufficient anchor points for a stable covalent enzyme immobilization.

3.2. *Efficiency of aminosilane loading on ceramic microbeads*

Amino-silanization with APTES is a widely used technique to achieve a sufficient amount of accessible NH_2-groups on the surface of inorganic materials and was needed for further covalent immobilization of GOx and CAT in the MBs.

First of all, in time dependent pre-experiments (0.5 – 20 h) we observed that the loading capacity of NH_2-groups/nm^2 of activated MBs revealed no relevant significant change after functionalization in 75 °C water-mix (route i) between 4, 8, 16 and 20 h (data not shown). Assuming, even with an overload of APTES precursor, the maximal loading is reached after 4 h of incubation. In general, with an increasing functionalization time undesired cross-linking of APTES molecules, especially when working in water-based solvents, is often seen. Therefore, further experiments were taken out only for 1, 4 and 20 h. Further, with long incubation times a mechanical damage of the MBs occurred and a certain material loss was noticed, basically due to shear stress while using a magnetic stirring fish. This could be avoided using a propeller driven stirring mode.

Fig. 5 presents the results of the photometrical AO-II assay indicating the amount of accessible NH_2-groups/nm^2 on the MBs for two APTES-functionalization routes (i = 75 °C water-mix, ii = 85 °C toluene under reflux) with and without surface hydroxylation. With longer functionalization time (1–20 h) a certain increase of NH_2-groups/nm^2 is determined for all strategies. Regarding the water-based route (i), the surface heat activation did not influence the loading procedure significantly. Certainly, the highest loading capacity of 0.024 NH_2-groups/nm^2 was revealed after 4 h silanization using activated MBs (A) under reflux in toluene (route ii). Though piranha activated MBs (P) showed only half of the APTES loading (0.013 NH_2-groups/nm^2). Route ii (A) using 4 h incubation was selected for all enzyme immobilization experiments.

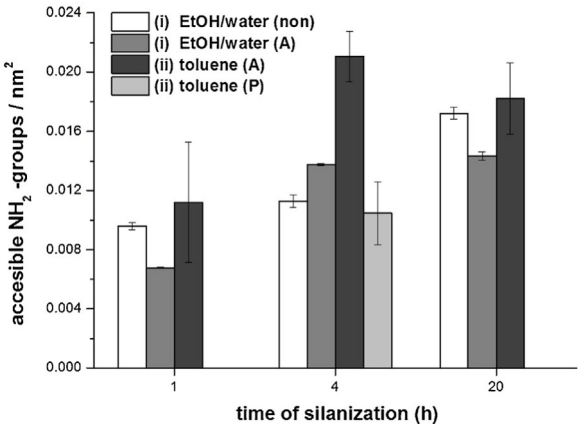

Fig. 5. Loading capacities of APTES-functionalized MBs varying both the hydroxyl-activation method and aminosilanization procedure (solvent, silanization time). Non-activated MBs and MBs activated by heat treatment (A) or piranha solution (P) were silanized via route (i) working in ethanol-water mixture at 75 °C or via route (ii) by refluxing in toluene at 85 °C. The experiments were performed in triplicates (n = 3). The accessible NH_2-groups were estimated performing the spectrophotometrical AO-II assay[31].

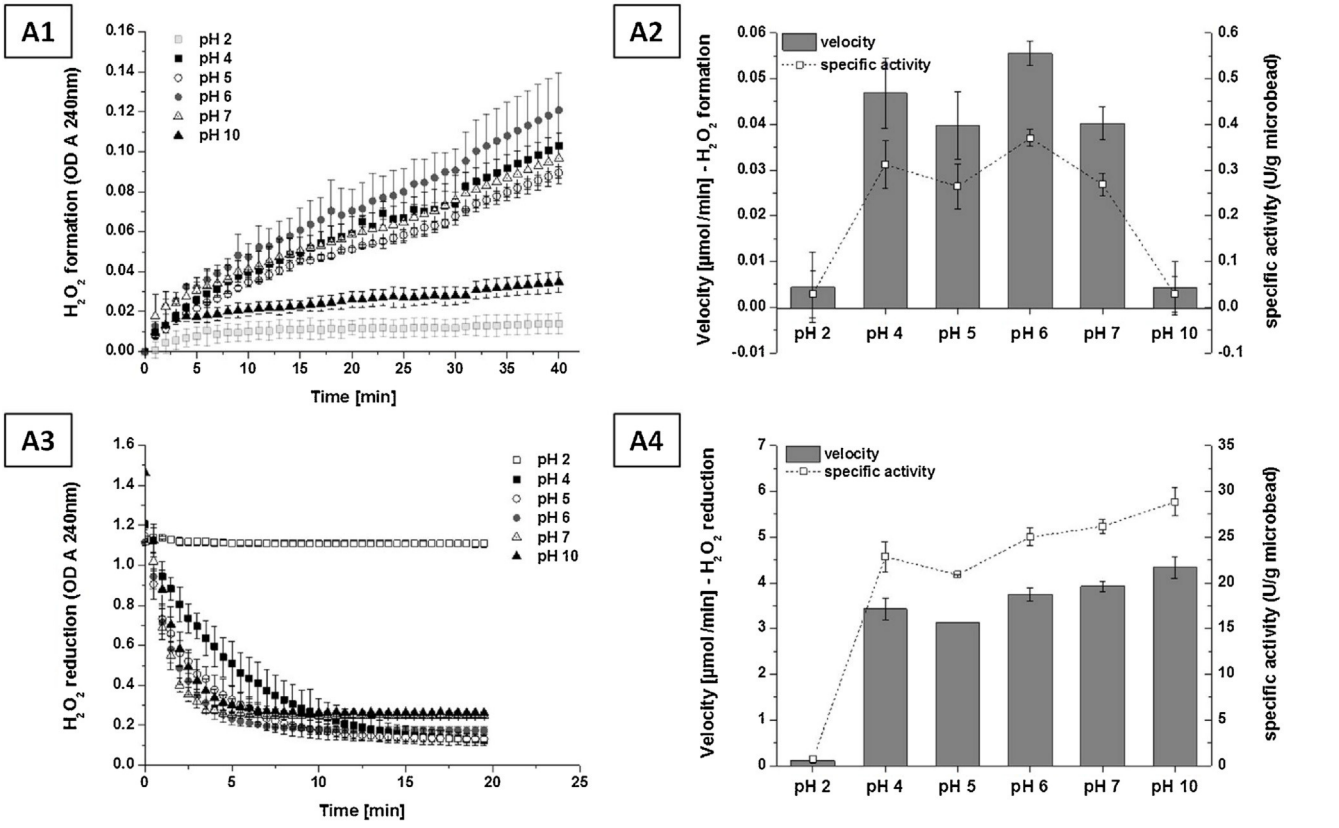

Fig. 6. Effect of the pH on the activity of immobilized GOx and CAT in batch mode: Spectrophotometrical analysis (λ = 240 nm) of the enzyme activities at 21 ± 2 °C (RT) following the H_2O_2 formation of GOx (A1, A2) and the H_2O_2 reduction of CAT (A3, A4) under different pH values in the solution. 1 Unit (U) = 1 µmol H_2O_2/min. The experiments were performed in triplicates (n = 3).

3.3. Activity of covalent immobilized GOx and CAT: single batch experiments

3.3.1. Determination of optimal pH and temperature

In order to determine the most suitable reaction conditions for immobilized GOx and CAT, their activity was analyzed in single batch experiments (Fig. 2), by changing the pH of the working solutions (pH 2–10, under RT) and by storing the MBs at different temperatures (4-70 °C, in pH 7). The catalytic activity is given as the absolute change of absorbance over time, resulting in the H_2O_2 velocity turnover and the specific enzyme activity (units per gram of MB). Fig. 6 presents the influence of the pH in the working solution on the enzymatic reaction of immobilized GOx (A1 and A2) and CAT (A3 and A4). In agreement with the literature [33,34] immobilized GOx had its highest activity between pH 4 and 7 only showing slight differences in the reaction slopes (A1), with a specific activity of around 0.3 ± 0.05 U/g of MBs (A2). Even in harsh acidic condition (pH 2) and basic condition (pH 10) a certain amount of enzyme activity was still detectable (A1). But in comparison with the results for neutral pH values the catalytic activity of immobilized GOx at pH 10 was strongly affected, losing 85% (0.03 U/g) activity. This behavior is mainly caused by breaking intra- and intermolecular bonds, changing the shape of the enzyme and leading to the dissociation of the subunits, therefore, reducing the enzyme effectiveness. In contrast, immobilized CAT had its highest specific activity at pH 10 converting 29 ± 1.6 U/g MB (A4). Below pH 4 only 2.5% activity was left, which was also reported by other working groups [36] as a result of acidic denaturation of the enzyme. Due to the fact that the standard substrate solutions were neutral right after preparation and because of the relative high activity of immobilized GOx and CAT at pH 7, the following temperature-related experiments were performed at pH 7. This condition aims at ensuring high enzyme activities during the envisaged tandem reaction.

The thermal stability of immobilized GOx remained in a wide range of incubation temperatures, presenting its highest activity at 4 °C and at RT (0.28 ± 0.02 U/g), as shown in Fig. S5 (A1 and A2). Unexpectedly, 17% of the enzyme activity (0.04 U/g MB) was left after incubation for 24 h at 70 °C. This could have been due to a so called "caging effect", when GOx might be shielded against thermal unfolding in the nano-pores of the MB, as it is described for nanocaged enzymes against protease digestion [37]. But this cannot be confirmed in this work. As suggestion for improvement, the thermal inactivation of GOx might be further inhibited in the presence of water-soluble polymers [38,39]. Unlike GOx, covalent immobilization did not stabilize the activity of CAT at high temperatures up to 70 °C (Fig. S5, A4). However, at 4 °C a specific activity of 22.8 ± 1.6 U/g was detected and even low activities remained after incubation at 50 °C (4.15 ± 0.07 U/g MB). Because of the high enzyme activities and in general to simplify the handling of the tandem reactor setup, for all further experiments ambient temperature (RT) was chosen as standard operating condition.

To analyze how the covalent immobilization of GOx and CAT affected the stability of the performance, the results were compared with the activities of the free enzymes in solution. Table S1 presents the specific activity of free GOx and CAT (U/mg enzyme) under different pH and temperature conditions where detailed information are given by Fig. S6 and Fig. S7. Free GOx had its highest activity at pH 4–7 and showed low activity in basic condition, similar to the immobilized state. An acidic environment of pH 2 resulted in a complete inactivation of free GOx, in contrast to the low but remaining activity of the immobilized enzyme. Free CAT showed an increasing enzyme activity with increased pH, showing its highest activity at pH 10 which is in agreement with immobilized CAT. It can be stated that for GOx the immobilization onto porous MBs had a positive effect and increased the temperature stability of the enzyme. After incubation at 70 °C 17% activity were left when normalized to 100% activity at RT, whereas the free GOx became inactive at 70 °C.

3.3.2. Estimation of enzyme loading on ceramic microbeads

Indirect quantification of the immobilized enzymes in the remaining solution at the end of the immobilization step was not possible due to the interference of NHS, EDC and their by-products with most conventional protein assays [40–43]. Thus, the amount of immobilized enzyme could only be estimated, by comparing the enzyme activities of immobilized and free enzymes directly, using the single batch setup (Fig. 2). An activity curve of free enzymes using different GOx (0.01 mg/ml–0.5 mg/ml) and CAT (0.0001–0.1 mg/ml) concentrations was obtained as shown in Fig.S8. In this way, the velocity of immobilized GOx and CAT was compared to the velocity of the free enzymes. Thus, immobilized GOx showed a turnover rate of 0.04 ± 0.004 μmol/min (Fig. S8A, as Δ) which resembles the slightly higher activity of 0.2 mg free GOx estimating at least 1.33 mg GOx/g MB (which is equal to 7.15×10^{-9} Mol = 4.3×10^{15} enzyme molecules). Similar conclusions were drawn comparing the immobilized CAT activity of 3.92 ± 0.11 μmol/min (Fig. S8B, as Δ) with the activity of free CAT. Thus, 1 g MB would be loaded with 0.033 mg CAT (which equals 1.3×10^{-10} Mol = 8.3×10^{13} enzyme molecules). The activity of free CAT did not increase with higher concentrations than 0.0025 mg/ml, due to an obvious substrate deficiency. Greater H_2O_2 amounts could have not been measured in the single batch set up, since they would exceed the quantification limit at 240 nm (Fig. S1). Nonetheless, these assessments at least give rise about the range of active enzyme on the carrier material.

3.4. Effect of different flow rates on the enzyme activity and stability in independent reactor compartments

Certain conditions as the pH of the surrounding solution and also the storage temperature have shown to influence the enzyme activity of the free as well as the immobilized GOx and CAT in single batch experiments (Section 3.3.1). Beyond that, other external conditions as the change of the continuous flow rate (6–360 ml/h) through the MBs used in the reactor setup (Fig. 3) can influence the enzymatic performance. In the first place the enzyme activity and the stability of the immobilized GOx and CAT over a streaming time of 24 h and secondly the overall performance of the immobilized enzymes itself (turnover rate of μmol H_2O_2/min/ml) was determined. Therefore, the reactor was equipped with GOx immobilized (E I) or CAT immobilized (E II) MBs and streamed with pH 7 buffered solution at constant temperature (RT), applying different flow rates between 0.1–6.0 ml/min for 24 h. Fig. 7 presents the flow rate dependent production of H_2O_2 by GOx immobilized MBs (A) and the consumption of H_2O_2 by CAT immobilized MBs (B). As expected, a higher applied flow rate led to higher volume throughput per time and thereby a higher overall substrate turnover was obtained. As shown in Fig. 7A, GOx immobilized MBs confirmed the hypothesis, because at time point zero 0.5, 1.4 and 6.0 μmol/min of H_2O_2 were produced with increasing flow rates (i.e. 0.1 ml/min, 1.0 ml/min, 6.0 ml/min). To directly compare these results, the mentioned reaction rates were normalized to a volume of 1 ml leading to H_2O_2 values for GOx of 0.47, 0.14 and 0.21 μmol/min/ml with increased flow rate. Indicating a lower activity per min at higher flow rates, this probably can be attributed to the fact that the substrate in the solution did not spent enough time in the reactor to reach all immobilized enzymes. With increased streaming time, especially at higher applied flow rates (0.1 ml/min > 1.0 ml/min > 6.0 ml/min) the production of H_2O_2 decreased. From 4 h to 24 h streaming time onwards at a flow rate of 1.0 ml/min, the activity of immobilized GOx remained almost unchanged, keeping more than 50% of its initial activity until the end.

Compared to immobilized GOx a similar trend was given for immobilized CAT (Fig. 7B). Initially (time point zero) immobilized CAT degraded 2, 20 and 106 μmol/min of the substrate H_2O_2 with

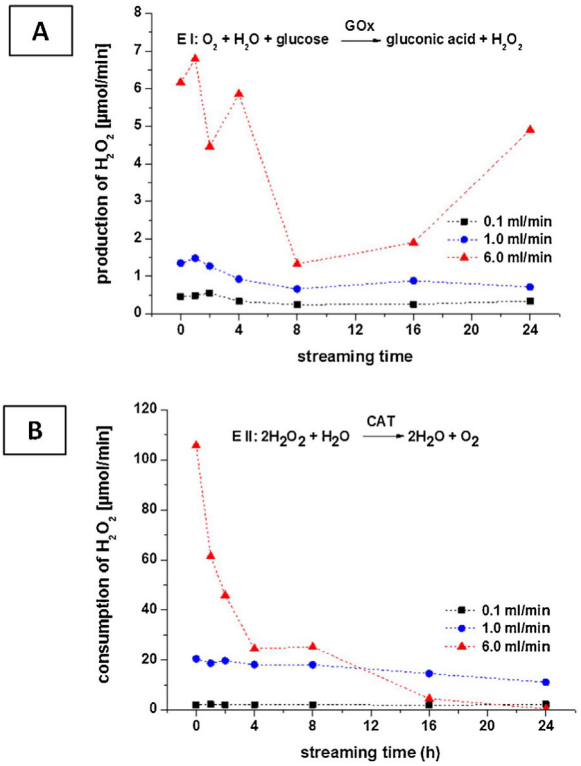

Fig. 7. Flow rate dependent performance of MBs immobilized with GOx (A) and CAT (B) in a flow reactor system at 21 ± 2 °C (RT) in buffered glucose solution (pH 7; 100 mM). Spectrophotometrical determination (λ = 240 nm) of the H_2O_2 production and consumption (µmol/min) of GOx and CAT, are given as function of the streaming time and flow rate. The results are calculated based on the applied reaction volume per min (i.e. for 0.1 ml/min = 0.1 ml, for 1.0 ml/min = 1 ml, and for 6.0 ml/min = 6 ml). The experiments were performed in triplicate (n = 3).

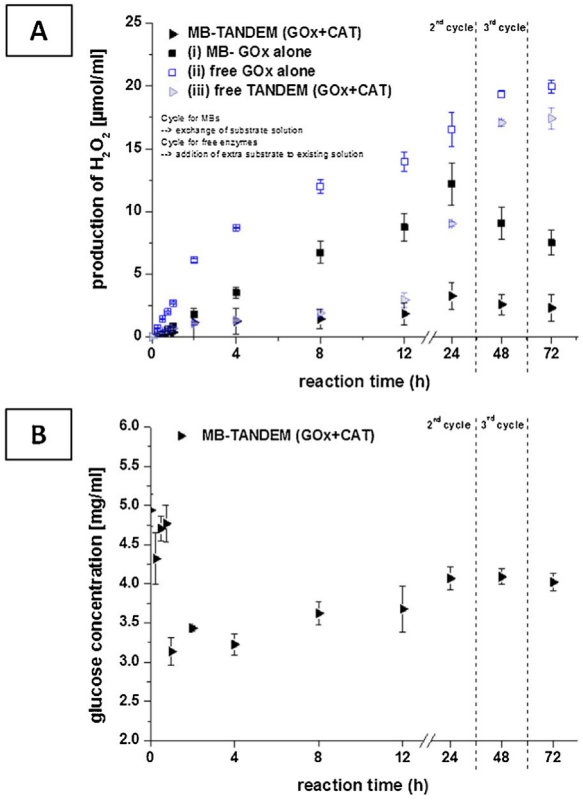

Fig. 8. Application of the tandem-flow reactor set-up in circulation filled with GOx- and CAT-functionalized MBs in the sequential compartments (EI and EII). The production of H_2O_2 (A) and the change in glucose concentration (B) was monitored over time for three operation cycles (feed) by spectrophotometrical determination (λ = 240 nm) and by using the *ACCU CHEK* Aviva sugar measuring device. Following references were analyzed: (i) 1.2 g MB-GOx alone (EI), (ii) free GOx (0.05 mg/ml) alone and (iii) free GOx (0.05 mg/ml) with free CAT (0.005 mg/ml) in tandem. The experiments were performed in triplicates (n = 3).

increasing flow rates (i.e. 0.1, 1.0 and 6 ml/min). For comparison, when normalizing the reaction values to a volume of 1 ml, a consumption of 1.9, 2.0 and 3.5 µmol H_2O_2 per min by CAT was obtained with increasing applied flow rate which shows a contrary effect compared to immobilized GOx. As expected, the loss of activity was time and flow rate dependent. After 24 h streaming time 100% CAT activity could be maintained at a low flow rate (0.1 ml/min), around 50% at an intermediate flow rate (1.0 ml/min) and only 0.3% at the highest applied flow rate (6.0 ml/min).

High liquid throughput implies strong external stress on the stability of the covalent immobilization of the enzymes and leads to wash-out effects with time. In case of GOx (Fig. 7A) at 6.0 ml/min a different phenomena was visible, as the overall activity tend to increase again over time. However, we assume that a higher level of dissolved oxygen is available for GOx by increasing the flow rate. Oxygen acts as a co-factor relevant for converting glucose to gluconic acid (glucolacton) and H_2O_2 (E I), therefore enhancing the activity. Knowing the impact of flow rate and streaming time on the activity of GOx and CAT, a reactor system with controlled activity and turn-over rates can be realized. To guarantee a good overall performance of GOx- and CAT-functionalized MBs in the tandem reactor system a flow rate of 1.0 ml/min was chosen as standard through flow condition.

3.5. Performance of GOx- and CAT-functionalized microbeads in the tandem flow reactor

To evaluate the tandem performance of immobilized GOx and CAT onto MBs, both compartments (EI and EII) of the tandem flow reactor setup (Fig. 3A) were filled with MBs immobilized with GOx- (estimated 1.33 mg total enzyme) and CAT (estimated 0.033 mg total enzyme). The tandem reactor has been used constantly for three cycles at a flow rate of 1.0 ml/min. For each 24 h cycle a feed of 30 ml fresh buffered glucose solution was used. For a better understanding of the tandem MB reaction, as reference (i) MB-GOx was used alone in compartment EI. Fig. 8A presents the overall production of H_2O_2 in the substrate solution and the mid-term stability over three cycles of performance, indicated by the dashed lines 2nd and 3rd feed. As expected, MBs solely immobilized with GOx (i) kept producing H_2O_2 on a high level resulting in a total amount of 366 ± 51 µmol (12 ± 2 µmol/ml) in the first cycle, 272 ± 38 µmol (9 ± 1 µmol/ml) in the second cycle and 225 ± 30 µmol (8 ± 1 µmol/ml) in the last cycle. Immobilized GOx (i) kept 75% of its activity after 48 h and even 61% after 72 h under constant use. This confirms a stable and active immobilization under dynamic flow conditions and an excellent operational stability. The general assumption that immobilized CAT would remove the produced H_2O_2 from solution when used in the tandem reactor was clearly stated as shown in Fig. 8A. Within the first hour of performance already 55% of the produced H_2O_2 was removed by immobilized CAT and a total of 79% after 24 h, overall this equals 98 ± 31 µmol (3 ± 1 µmol/ml) which remained in the solution. It can be seen that the MB tandem system kept its catalytic activity over two more cycles as the level of H_2O_2 reduction was still reduced by ~70% after 48 and 72 h, respectively. That, in turn, is an argument for a strong and active immobilization of CAT, otherwise an increasing H_2O_2 concentration would have been expected.

In order to present the actual benefit of immobilized GOx and CAT in the tandem reaction, the results were compared to the free state of the enzymes (Fig. 8A), which are likely to be less stable in solution over time. Following reference conditions were tested in the reactor under constant flow: (ii) free GOx alone in solution and (iii) free GOx with free CAT in tandem in solution. Thus, 30 ml buffered glucose solution was equipped with the enzymes (ii or iii) and the reaction was monitored for 24 h. Then fresh glucose substrate was added into the same solution to run the 2nd and 3rd cycle (feed). Using free GOx (ii) in the reactor the H_2O_2 concentration was rising constantly up to 496 ± 41 μmol (17 ± 1 μmol/ml) after the first cycle. The second and third cycle only resulted in a further increase of 42 ± 9 μmol (1.40 ± 0.3 μmol/ml) and 19 ± 15 μmol (0.62 ± 0.5 μmol/ml) in total, indicating the insufficient usability of free GOx under constant flow longer than 24 h. An inhibitory effect of the rising H_2O_2 concentration on the activity of GOx cannot be excluded. A significantly lower H_2O_2 concentration level, attributed to the sequential consumption of H_2O_2 by free CAT, was monitored using the free tandem set up (iii). In this case the same performance of CAT could not be detected for more than 24 h. The drastically increased H_2O_2 concentration within the 2nd and 3rd cycle implied a total inactivity of free CAT. The immobilized enzymes certainly achieved a better overall tandem reaction and provided benefits in the mid-term stability and reusability.

Furthermore, to confirm the reduction of the substrate in the solution over time, when using the MBs immobilized with GOx and CAT in the tandem system (Fig. 8B), the glucose concentration was monitored via an Accu Check Aviva device. The initial glucose concentration was reduced by 35% within 4 h, and started to increase again to 82% of the starting concentration after 24 h. A similar result was found for the 2nd and 3rd cycle. It can be excluded, that those results originate from leaching enzymes, since even in high concentration (0.5 mg/ml) GOx and CAT itself did not show a significant response to the assay. Furthermore the assay did not show a sensitivity towards the byproduct H_2O_2, the linker molecules NHS and EDC, which may also be ignored. Furthermore, bacterial contamination could also be excluded, since no cells were found in the solution over time. However, the risen glucose concentration over time might be caused by the intermediate product gluconic acid (glucolactonate), which tends to interfere to a certain level with the Accu Check Aviva device and other enzyme based glucose assays. However, it can be summarized, that the MB tandem system was successfully build-up and efficient in reducing the overall glucose concentration and in degrading the produced H_2O_2 in solution over time and under repeating cycles.

4. Conclusion

In this study, we fabricated porous MBs (Al_2O_3/SiO_2) covalently immobilized with GOx and CAT, estimated the enzyme amount per mass (1.33 mg GOx per g of MB and 0.033 mg CAT per g of MB) and evaluated their catalytic activity under different pH and temperature conditions by comparing to the free enzymes using simple spectrophotometric analysis. Generally, both immobilized enzymes showed a relative high activity at pH 7 and 21 ± 2 °C which is beneficial for real applications. The immobilization lead to an increased activity in acidic and basic conditions as well as towards 70 °C incubation.

Finally, we demonstrated the efficient and controllable application in a tandem flow reactor system. The general assumption that immobilized CAT would remove the produced H_2O_2 from solution (EII: $H_2O_2 \rightarrow H_2O + O_2$), when used under flow conditions in the tandem reactor with immobilized GOx (EI: glucose $\rightarrow H_2O_2$) was clearly stated as shown in Fig. 8A. Up on that findings, there were additional interesting details of the tandem system – (i) a flow rate (0.1–6.0 ml/min) dependent performances showing the higher the flow the greater the H_2O_2 production up to 6 μmol/min (GOx) and consumption up to 106 μmol/min (CAT). (ii) a long term stability over 24 h (1 ml/min), but a reduced overall performance at relative high flow rate conditions (6.0 ml/min) probably indicating an enzyme leaching; (iii) exceptional benefits in the mid-term stability and reusability over three cycles (72 h), compared to the free states of the enzymes, which were highly unstable in solution over time periods greater than 24 h; (iv) the overall glucose concentration was reduced to a certain level, and then started to increase again, interference of the intermediate product gluconic acid (gluconlactone) is possible.

To help isolating the uncertainty of the mentioned findings, interesting as well as critical points should be addressed in future work – (i) non-enzymatic glucose assay and HPLC analysis is of interest; (ii) to overcome the quantification issue a different immobilization strategy could be considered e.g. a straightforward click-chemistry approach (iii) factors influencing the overall performance, as internal diffusion, as well as the uniformity of enzyme distribution inside the material, could be addressed for a deeper understanding of reduced or enhanced reaction rates [15,19]; (iv) a greener and resource efficient production, using different sources of SiO_2 from diatoms as well as clay is of interest and (v) the application in special loop reactors could be intended, as the MBs are buoyant under multiphase flow conditions. The findings of this feasibility study serve to bring the versatile porous material "microbead" in focus, when enzyme immobilization strategies are in need of an adaptive set up.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2017.06.015.

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