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Glucose level determination with a multi-enzymatic cascade reaction in a functionalized glass chip†

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In this work we show the functionalization of the interior of microfluidic glass chips with poly(2-hydroxyethyl methacrylate) polymer brushes as anchors for co-immobilization of the enzymes glucose-oxidase and horseradish peroxidase. The formation of the brush layer and subsequent immobilization of these enzymes have been characterized on flat surfaces by atomic force microscopy and Fourier transform infrared spectroscopy, and studied inside glass chips by field emission scanning microscopy. Enzyme-functionalized glass chips have been applied for performing a multi-enzymatic cascade reaction for the fast (20 s) determination of glucose in human blood samples and the result is in excellent agreement with values obtained from the conventional hospital laboratory. The limit of detection of this bi-enzymatic method is 60 μM. With the advantages of high selectivity and reproducibility, this functionalization method can be used for improving the efficiency of glucose sensors.

1 Introduction

Performing biocatalytic processes with immobilized or dissolved enzymes in Lab-on-a-Chip devices has been shown to lead to better yields and reduced consumption of reactants and catalysts, with additional advantages offered by the integration of analytical functionality.[1–3] The advantages of attaching enzymes within microfluidic reactors have been demonstrated by applying different immobilization procedures, either to the surface interior of the channel network or to packed beds loaded in such microfluidic systems.[1,4–7] Sequential (cascade) enzymatic processes are increasingly applied for industrial chemical synthesis[8–10] as well as for medical diagnostics.[11] Performing complex bioprocesses, such as one-pot *in vitro* cascade reactions, in microfluidic chips with immobilized multiple enzymes leads to systems with high process automation and reproducibility. In fact, it is envisioned that properly designed chips with immobilized enzymes allow the detection and quantitative determination of biomarkers that are characteristic for a specific pathological condition. This opens a route towards the development of low-cost, portable (perhaps handheld) point-of-care instruments for rapid and reliable diagnosis in the field of healthcare and medicine.

Applications could be for example early diagnosis (and follow up) of chronic disease by monitoring metabolic biomarkers in body fluids. Eventually such devices could be combined with automatic microdosing systems that dispense a proper medicine.

Monitoring of the glucose level in blood (glycaemia) is crucial for the dosage of insulin given to patients suffering from diabetes. Blood glucose home testing devices are used daily by diabetic people for self-monitoring. The majority of these devices rely on disposable screen-printed enzyme electrode test strips based on redox-couple-mediated enzymatic oxidation of glucose with either glucose oxidase (GOx) or glucose dehydrogenase (GDH).[12–14] Although the clinical accuracy of these systems is demonstrated by the multitude of commercially available types, such electrochemical systems suffer from interferences of some species present in the body (*e.g.* ascorbic acid, uric acid) and electrode fouling when in contact with the samples.[12,15] Attempts to overcome these problems are focused on the development of electrochemical devices based on microfluidic chips.[16–20] Alternative to electrochemical detection, glucose levels can be monitored through an enzymatic cascade reaction. In this reaction GOx oxidizes glucose to gluconolactone producing hydrogen peroxide. The generated H_2O_2 activates the enzyme horseradish peroxide (HRP), which oxidizes a light adsorbing dye molecule that can be detected by photometric based spectroscopic measurements.[15,21–24] The GOx–HRP bi-enzymatic reaction has also been utilized for determination of the glucose level by an electrochemical

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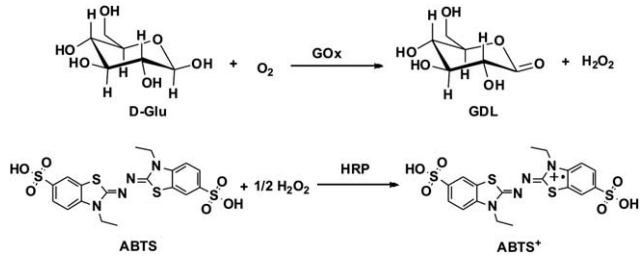
detection system.[25] These studies demonstrate that cascade reactions, where an enzyme is catalytically linked to another enzyme, can produce signal amplification and thereby an increase in the efficiency of biosensors. However, the applied immobilization procedures for the enzymes (*i.e.* physical adsorption on macroporous silica foam[24] or electrostatic and hydrophobic absorption on a polyaniline electrode surface[25]) caused a partial inactivation of the HRP (thus reduction of its activity),[25] or co-confinement in nanospaces impacted the catalytic activity of the enzymes.[24]

Unlike electrochemical glucose meters, photometric systems did not receive too much attention over the past years. Although the specificity of GOx–HRP bi-enzymatic reaction for glucose determination has been demonstrated,[26,27] the main problems of these systems are related to the instability of the enzymes,[28,29] and uncertainty of the measurement data due to the large enzymatic area necessary for the photometric detection.[30] Nowadays, miniaturization trends in analytical chemistry have led to the development of Lab-on-a-Chip devices which allow simultaneous sample treatment and photometric measurement with high accuracy and precision.[31–33] An example of using such a chip-based system for glucose measurements has been reported,[15] with the aim to improve detection specificity and reduction of interference with other species present in the body. Until now, most reported enzyme-functionalization procedures for Lab-on-a-Chip devices for enzymatic cascade reactions for detection of glucose (or others biomarkers) have been based on separation of microfluidic channels in various compartments by integration of valves,[34–36] or on patterning of a capillary into different enzymatic zones by means of shadow-mask lithography.[37–41]

In this study we demonstrate co-immobilization of the enzymes GOx and HRP as a much simpler way to achieve a glucose sensor: GOx and HRP are randomly co-immobilized on the interior of a microfluidic channel network of glass chips using poly(2-hydroethyl methacrylate) (PHEMA) polymer brushes as anchors.[42,43] Polymer brushes[44] have been used to immobilize enzymes or other catalysts on the wall of microfluidic systems.[45–47] The advantage of this approach is that the amount of catalyst/enzyme can be tuned by varying the polymerization time, which makes this platform adaptable to different requirements. Moreover, it has been shown that lipase enzymes, immobilized onto these polymer brushes, are extremely stable in terms of lifetime and their catalytic activity is identical to that of the same enzyme in solution.[47]

In this work, GOx + HRP functionalized chips are used for the determination of the concentration of glucose in standard solutions and human serum by means of a cascade enzymatic reaction: D-glucose (D-Glu) is oxidized to δ-D-gluconolactone (GDL) and hydrogen peroxide (H_2O_2) by glucose-oxidase (GOx) (Scheme 1). The H_2O_2 diffuses to the HRP binding site where oxidation of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) occurs. The concentration of the oxidized form of ABTS ($ABTS^+$) is proportional to the concentration of D-Glu and can be easily quantified by UV-vis detection. With the aim to test the cascade reaction in enzyme-functionalized chips, they were coupled in-series with a small volume in-line UV-vis detection cell. Measurements of the glucose concentration performed



Scheme 1 Multi-enzymatic cascade reaction involving glucose-oxidase (GOx) and horseradish peroxidase (HRP).

with standard solutions and human serum demonstrated that the immobilization procedure has no influence on the enzymatic activity.

Moreover, such measurements were conducted within 20 s, and display high selectivity, reproducibility and long-time stability, which open the way for the development of more efficient glucose biosensors and can be considered as a first step towards portable point-of-care device for healthcare and medical treatments.

2 Experimental

2.1 Reagents and equipments

All reagents were purchased from Aldrich Chemicals. 2-Hydroxyethyl methacrylate (HEMA) was distilled prior to use, whereas the other chemicals were used without further purification. 2-Bromo-2-methyl-propionic acid 3-trichlorosylanyl-propyl ester was synthesized following a reported procedure.[48] Methanol (analytical reagent grade) was used without further purification, while toluene was distilled over sodium. Water was purified with a Milli-Q pulse (MILLIPORE, $R = 18.2$ MΩ cm) ultra-pure water system.

The thickness of PHEMA layers at various functionalization steps was measured using atomic force microscopy (AFM; Bruker AXS, Germany) on flat surfaces (see ESI† for details). Also, FTIR spectroscopy was used to investigate the immobilization and functionalization procedure. FTIR spectra were recorded using a Shimadzu IR Prestige 21 spectrometer. The spectra of brushes were taken in transmission mode using a bare silicon wafer as the background (see ESI† for details).

Blank and functionalized chips were analysed by cross-sectional analysis with Field Emission Scanning Electron Microscopy (FESEM). FESEM images were recorded with an Auriga 405 system (Zeiss), using low operating voltage and current conditions, to eliminate charging effects and to avoid material damage.

2.2 Glass chips and set-up

Chips of 20 mm × 10 mm containing serpentine-shaped channels with a total length of 175 mm, and a (nearly) half-circular cross-section with a depth of 50 μm and a width of 110 μm (reaction volume ~1.5 μL). The procedure for the fabrication of these chips was described elsewhere.[46,49] For all the experiments, the chips were positioned in a home-built chip

holder (Delrin) designed for fitting fused silica fibers into the inlet/outlet chip reservoirs by means of commercially available Upchurch NanoportTM assembly parts. Sample solutions were pressure-driven mobilized by means of a PHD 2000 series syringe pump (Harvard Apparatus, United Kingdom) equipped with 250 μL flat tip syringes (Hamilton). Syringes were connected to fused silica capillaries (360 μm o.d., 100 μm i.d.) by means of Upchurch NanoportTM assembly parts (*i.e.* NanoTightTM unions and fittings, Upchurch Scientific Inc. USA). The temperature of the chips was controlled by interfacing a thermoelectric module with a heat sink to the chip. The temperature variation on the glass surface of the chip measured with a thermocouple was less than ±0.1 °C. For monitoring the enzymatic reactions, an in-line UV flow cell (Zeutec GmBH, Germany) was mounted in series with the chip. This flow-through cell, with a spectral UV/Vis/NIR range of 250–1100 nm, has an optical path length of 5 mm, and an internal volume of 1 μL. By means of two optical fibers (Ocean optics Inc., The Netherlands) this cell was connected to a deuterium–halogen light source (DT-Mini-2-GS, Mikropack GmbH, Germany) and a fiber optic spectrometer (HR4000, Ocean optics Inc., The Netherlands).

2.3 Polymer brushes: immobilization and functionalization

Prior to immobilization and functionalization in flow channels of chips, the procedure was performed on microscope glass slides and oxidized silicon substrates, of which the details are given in the ESI.† In the case of chips, a Piranha solution was flushed through the microfluidic channels and kept inside for 10 min to activate the OH-groups. The channel was then thoroughly rinsed with Milli-Q water at a flow rate of 5 μL min^{-1} for 10 min and dried with a stream of nitrogen. Afterwards, a solution of 0.2% of 2-bromo-2-methyl-propionic acid 3-trichlorosylanyl-propyl ester in toluene was flowed through the channel (one night; 0.1 μL min^{-1}), subsequently the channel was rinsed with toluene and dichloromethane (10 min; 1 μL min^{-1}), and dried with a stream of nitrogen. Post this step, a degassed water solution of HEMA, 2,2′-bipyridyl, copper(I) chloride and copper(II) bromide was flowed in the chip and kept inside under no-flow conditions for 90 min in order to form the PHEMA brush film.

Afterwards, the channel was rinsed with methanol and water (10 min; 1 μL min^{-1}) to completely remove the copper residues, and dried with a nitrogen flux. A THF solution (2 mL) of succinic anhydride (100 mg) and triethylamine (100 μL) was flowed through the chip (overnight; 0.1 μL min^{-1}; 60 °C), followed by rinsing with THF and water (10 min; 1 μL min^{-1}). After this step, a water solution (1 mL) of *N*-hydroxysuccinimide (NHS) (13 mg) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (75 mg) was flowed in the channel (0.1 μL min^{-1}; 1 h), followed by rinsing with demineralized water. Next, a phosphate buffer solution (10 mM, pH = 7.5) containing GOx and HRP (1 mg mL^{-1}) was flushed through the chip (8 hours; 0.1 μL min^{-1}) in order to immobilize GOx and HRP onto the activated brushes anchored inside the channel. Finally, the channel was rinsed with phosphate buffer for 30 min (1 μL min^{-1}), in order to remove all enzyme molecules that did not link covalently to the polymer film.

2.4 Enzymatic cascade reaction in chips

The progress of the cascade enzymatic reaction was monitored at 735 nm, where $ABTS^+$ shows a maximum absorption peak. The change in absorbance was converted to the concentration of $ABTS^+$ using an extinction coefficient (ε) of 3.8×10^4 M^{-1} cm^{-1} (calculated from an $ABTS^+$ calibration curve).

For initial rate experiments, D-Glu solutions (0.3–9.6 mM) and ABTS (40 mM) in buffer (10 mM, pH = 7.5) were flowed through the chip at various flow rates ranging between 1 and 40 μL min^{-1}. The v_i values were calculated by least-squares fitting the equation $[ABTS^+] = v_i t$ to the experimental data, while k_{obs} was calculated by fitting the equation $v_i = dp/dt = k_1[\text{D-Glu}][ABTS] = k_{obs}[\text{D-Glu}]$ to the data. The experiments were performed using three different chips (identically functionalized), and for each chip the measurements were repeated at least 3 times. The limits of detection (LOD) and quantification (LOQ) were calculated using the standard deviation (σ^B) of the buffer solution signal: $LOD = 3 \times \sigma^B_{vi}/k_{obs}$ and $LOQ = 10 \times \sigma^B_{vi}/k_{obs}$.

D-Glu in human serum was measured by flowing the serum solution without any treatment in the chip and a 40 mM ABTS solution in a phosphate buffer (10 mM, pH = 7.5). These experiments were performed using three different chips and for each chip the measurements were repeated 3 times. For comparison, the amount of D-Glu in serum was also analysed in an approved laboratory for blood tests (www.usi.it).

The amount of GOx and HRP enzymes in the interior of the chips was quantified using a bicinchoninic acid assay (BCA) protein assay kit (Thermo Scientific). Several standard samples were prepared with a known concentration of BSA (bovine serum albumin) standard protein, and with these standards a calibration curve was determined (by plotting the BSA concentration *versus* the absorbance at 562 nm as measured for each standard sample). A working reagent solution was pumped into the chip and kept inside for 30 min at 37 °C. Subsequently, the chip was rinsed with buffer, and the eluted solution collected with a Hamilton syringe and injected into the flow cell. By measuring the absorbance at 562 nm using the calibration curve, the total concentration of the enzymes was determined. This was performed with three separately functionalized chips (the same procedure applied to each chip).

3 Results and discussion

3.1 Polymer brushes: immobilization and functionalization onto oxidized silicon and glass surfaces

In order to verify the reaction conditions for immobilization of GOx and HRP on the walls of the microchannel in chips, the procedure was first investigated on glass slides. PHEMA polymer brushes were fabricated on glass surfaces *via* atom transfer radical polymerization (ATRP) (Fig. 1a and b). Afterwards, the PHEMA polymer film was treated with succinic anhydride (SA) and *N*-hydroxysuccinimide (NHS) in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to obtain NHS-ester groups (PHEMA-SA-NHS) (Fig. 1c and d).[42,47] Then a GOx and HRP phosphate buffer solution was poured on the PHEMA-SA-NHS polymer coated surfaces. The enzymes are randomly

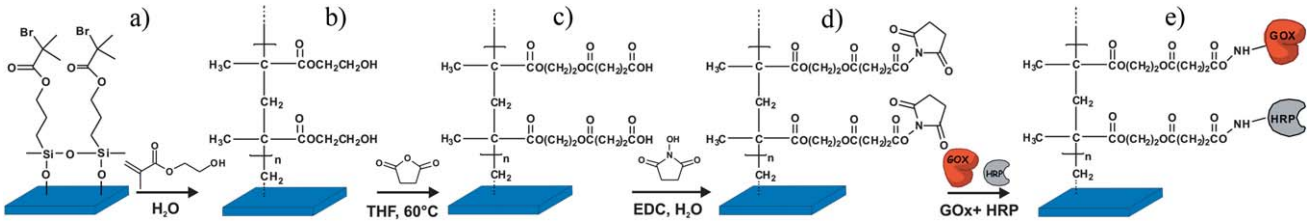


Fig. 1 Procedure for enzyme immobilization onto glass surfaces: (a) initiator monolayer, (b) PHEMA, (c) PHEMA functionalized with SA (PHEMA-SA), (d) PHEMA functionalized with NHS (PHEMA-SA-NHS), and (e) PHEMA with co-immobilized enzymes (PHEMA-GOx + HRP).

co-immobilized onto the polymer layer (Fig. 1e) through formation of amide bonds between the amino groups of the amino acids of the enzymes and the NHS-ester moieties (PHEMA-GOx + HRP). Each step of the surface functionalization sequence was analysed by atomic force microscopy (AFM) to investigate the thickness of the polymer film, of which images are given in the ESI† and numerical data in Table 1.

AFM analysis indicated that PHEMA films have a thickness of 37 nm after a polymerization time of 90 minutes. After the reaction of PHEMA films with SA and NHS, the thickness increased up to 56 nm (PHEMA-SA) and 70 nm (PHEMA-SA-NHS), which suggest that PHEMA was successfully functionalized. PHEMA-SA-NHS films were treated with buffer solutions of GOx or HRP, and with a mixture of both enzymes. In the presence of the enzymes, film thicknesses increased to values up to 100 nm, showing that both HRP and GOx are successfully immobilized onto the polymeric film (Table 1). AFM was also performed on PHEMA-SA-NHS and PHEMA-GOx + HRP films in a liquid environment, by wetting the films with a drop of phosphate buffer solution (pH = 7.5, 10 mM). Data are reported in Table 1, accompanying images in the ESI.† Under these wet conditions the measurements displayed a thickness of 93 and 900 nm for PHEMA-SA-NHS and PHEMA-GOx + HRP respectively (Table 1), confirming the immobilization of GOx and HRP in the polymer film, and also that the enzymes are involved in the swelling mechanism of the brush film when wetted with the phosphate buffer solution. Additional evidence of enzyme-functionalization of the brush layer was obtained by FTIR spectroscopy (see ESI†), which confirmed the successful co-immobilization of the HRP and GOx enzymes.

3.2 Polymer brushes: immobilization and functionalization in chips

The procedure to functionalize glass surfaces was applied to the interior of glass chips (Fig. 2). In order to verify the immobilization of the polymer layer on the channel interior, after applying the functionalization procedure, one chip was cleaved and cross-sections of the channel were analysed by field emission scanning electron microscopy (FESEM). The thickness of the brush film on the walls of the channel was 80 ± 10 nm (Fig. 3). The total concentration of enzymes immobilized onto the brush layers in chips was quantified using a BCA assay,[50] and found to be $(3.78 \pm 0.39) \times 10^{-8}$ μg μm^{-2}.

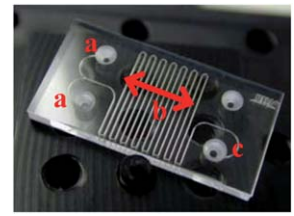


Fig. 2 Microfluidic chip: (a) inlets, (b) reaction zone, and (c) outlet.

3.3 Enzymatic cascade reaction in chips

Enzyme-functionalized chips turned out to be highly efficient for on-chip mixing of the reacting solutions and down-stream catalysis. No reaction was measured when the reagents were flowed through a chip with non-functionalized PHEMA brushes, evidencing that the enzymes are the catalytic species.

Chips with GOx + HRP functionalized polymer brushes were used for the determination of glucose levels in water and human serum. The D-Glu concentration was measured by using the initial rate method applying an excess of ABTS. Solutions of D-Glu and ABTS were flushed into the chip at various flow rates in the 1–40 μL per min-range, by means of which the reaction time could be controlled. The plot of reaction time *vs.* $ABTS^{+}$ concentrations (Fig. 4a) allows calculation of the initial rates (v_i) for D-Glu concentrations (0.3–9.6 mM). Fig. 4b shows each v_i as a

Table 1 Thicknesses of the films after each functionalization step as measured with AFM (in air and buffer)

Sample	Thickness (in air) [nm]	Thickness (in phosphate buffer, 10 mM) [nm]
PHEMA	37 ± 1	—
PHEMA-SA	56 ± 2	—
PHEMA-SA-NHS	70 ± 6	93 ± 5
PHEMA-GOx	101 ± 4	418 ± 7
PHEMA-HRP	107 ± 8	530 ± 15
PHEMA-GOx + HRP	103 ± 6	900 ± 27

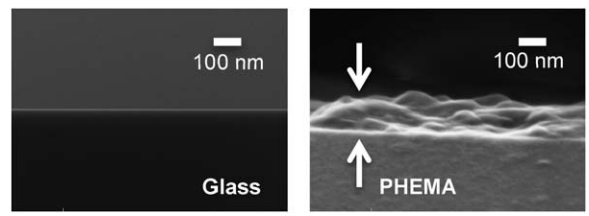


Fig. 3 Cross-sectional FESEM images of a blank glass microfluidic channel (left), and a PHEMA-GOx + HRP functionalized channel (right).

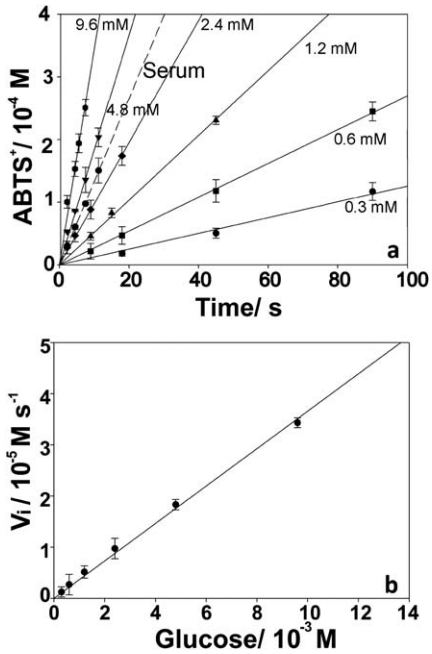


Fig. 4 (a) Formation of $ABTS^+$ catalysed by the enzyme-functionalized polymeric coating in the microfluidic channel for different concentrations of D-Glu (0.3–9.6 mM, solid lines), and for serum solution (dashed line), and (b) initial rates (v_i) *vs.* glucose concentrations.

function of the D-Glu concentration. The linear trend clearly indicates a first order reaction in D-Glu. A least-squares procedure was used to fit the data points in Fig. 4b, yielding a value of $(3.65 \pm 0.2) \times 10^{-3}$ s^{-1} for k_{obs}.

The enzymatic chip can also be used to determine the unknown D-Glu concentration in a sample, just by performing the enzymatic cascade reaction and measurement of v_i and use of k_{obs}. Thus, Fig. 4b can be considered as the calibration curve of this analytical method and the sensitivity of this method equals the slope of the calibration curve (*i.e.* it corresponds to the value of k_{obs}: *ca.* 3.65×10^{-3} s^{-1}). With this approach unknown concentrations of D-Glu can be determined within *ca.* 20 s (Fig. 4a), without waiting for complete oxidation of the D-Glu, nor a reference reading.

The limits of detection (LOD) and quantification (LOQ) for this chip-based method are found to be 60 μM and 200 μM, respectively.

The above-reported experiments were carried out with D-Glu concentrations including the range as present in human blood (3–6 mM). Therefore, this procedure was also applied to human serum with an unknown glucose level. Such an experiment shows the capability of these chips for biomedical applications. Solutions of ABTS and serum were flowed through a functionalized microfluidic channel. The formation of $ABTS^+$ occurred within a few seconds (Fig. 4a), which allowed fast determination of v_i. A v_i-value of $(1.32 \pm 0.07) \times 10^{-5}$ M s^{-1} was found, which corresponds to a glucose concentration of $(3.62 \pm 0.18) \times 10^{-3}$ M. Analysis of this human serum by conventional hospital laboratory equipment yielded a glucose concentration of $(3.44 \pm 0.05) \times 10^{-3}$ M, showing excellent agreement with measurements performed with the functionalized chips.

The reproducibility of the procedure for co-immobilization of GOx and HRP in microfluidic chips is very high, as evidenced by the (nearly) similar total concentration of enzymes in various separately functionalized chips. This reproducibility is also highlighted by the data obtained for the bi-enzymatic cascade reaction performed in separately functionalized chips as well as in repeatedly used chips, which shows the very high degree of intra- and interchip reproducibility. In fact, the enzymes did not show a loss of activity nor leaching during the glucose determination: a set of experiments showed that these PHEMA functionalized chips can be (re-)used up to one month (*ca.* 120 working hours) without any decrease of the enzymatic activity, which proves that the PHEMA-GOx + HRP film is a very stable nanostructured catalytic material.

4 Conclusions

In conclusion we have shown that by using functionalized PHEMA-brushes in glass chips it is possible to determine glucose concentrations of standard solutions and human serum by using an enzymatic cascade reaction. The enzymes GOx and HRP were randomly co-immobilized onto polymeric brushes grown on the interior of glass microfluidic networks. Chip-based enzymatic measurements of the glucose concentration in human serum are identical to values obtained with standard procedures. The advantage of this Lab-on-a-Chip approach is that the functionalization procedure does not require additional steps for spatial separation of the enzymes such as patterning or implementation of valves.[34–41] Moreover, the small volume of the chips results in fast analysis times (<20 s). It is remarkable to note that the co-immobilization of GOx and HRP in microfluidic channels does not affect their functionality: the enzymes do not show a loss of activity nor leaching during the glucose determination, are (very) stable in time and reusable. This method can be extended to other enzymes for the determination of biomarkers correlated with other diseases (such as cholesterol[51] and triglycerides[52]). Multi-enzymatic chips with integrated detection methods,[53] such as amorphous silicon sensors,[54,55] fasten the development towards (home) point-of-care, easy-to-use health monitoring devices, and will be part of our further research.

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