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Simple enzyme immobilization inside glass for enzymatic cascade reactions

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1. Polymer and protein surface density determinations with the TInAS

During a measurement, the TInAS device records the interference spectra consisting of secondary fringes.[1] From these patterns, the optical path Ω_A can be calculated. Independent on the type of molecule adsorbed, the TInAS software was set to use the refractive index of dry polystyrene ($n_A = n_{Polystyrene} = 1.450$) for all adsorbed layers to determine the raw layer thickness D_A (output data). To transform this raw output data into effective dry adsorbed mass m_A, first the real layer thickness D_{rA} has to be calculated using Equation S1, where n_A is the correct refractive index of the corresponding adsorbate A (polymer or protein).

$$D_{rA} = \frac{\Omega_A}{n_A} = \frac{D_A \cdot n_{Polystyrene}}{n_A} \qquad \text{(S1)}$$

Then the De Feijter equation[1,2] (Equation S2) is used to calculate m_A:

$$m_A = \frac{n_A - n_M}{dn_A / dc_A} \cdot D_{rA} \qquad \text{(S2)}$$

where n_A - n_M is the refractive index difference between the adsorbate (n_A) and the medium $(n_M = n_{Water} = 1.337)$, and dn_A/dc_A the concentration dependence of the refractive index of the adsorbate. Finally the density of molecules present on the surface Γ_A can be calculated using Equation S3:

$$\Gamma_A = \frac{m_A}{M_A \cdot S} \qquad \text{(S3)}$$

where M_A is the molar mass of the *de*-PG2 repeating unit or of the protein, and S the surface area on which the adsorption occurred (34.5 mm^2). Values for n_A, dn_A/dc_A and M_A used for B-*de*-PG2, avidin and B-GOD are given in Table S1. For all measurements $n_{SiO2} = 1.462$ was set.

Table S1. Some of the properties of B-*de*-PG2, avidin and B-GOD

	B-*de*-PG2	Avidin	B-GOD
M_A of repeating unit or protein (g/mol)	822.99	66'000	153'000[a]
n_A (-)	1.52[b]	1.45[b]	1.45[b]
dn_A/dc_A (cm^3/g)	0.140[b]	0.182[c]	0.182[c]

[a] from the Toyobo product catalogue; [b] from Fornera *et al.*[3]; [c] from Vörös[4]

2. GOD quantification in solution

The concentration of active GOD in solution can be quantified *via* the amount of glucose that can be oxidized during a certain time span, using the cascade reaction system GOD/HRP with D-glucose/OPD as substrates, as sketched in Figure 1b. The activity measurements were carried out in phosphate solution pH 5.0, 0.15 M NaCl. At these conditions, the formed reaction product 2,3-diaminophenazine (DAP) has maximum absorption at 427 nm (λ_{max}), and it can be easily quantified spectrophotometrically similarly as we reported for the HRP/OPD system at pH 7.2.[5]

In a 5 mL glass vial containing 3.14 mM OPD, 3.45 mM D-glucose, and 1.98 nM HRP (V_{TOT} = 3000 – 2800 μL), 0 - 200 μL of a 15 nM GOD solution were added. After incubation in the dark for 30 min, the UV-visible spectrum (between 325 and 600 nm) of the solutions was recorded, see Figure S1a. After subtraction of the blank value obtained in absence of GOD, the GOD concentration was plotted *versus* the absorbance at 427 nm. A_{427nm} was linearly dependent on GOD at least between 50 and 1000 pM (= 1 nM), as shown in Figure S1b.

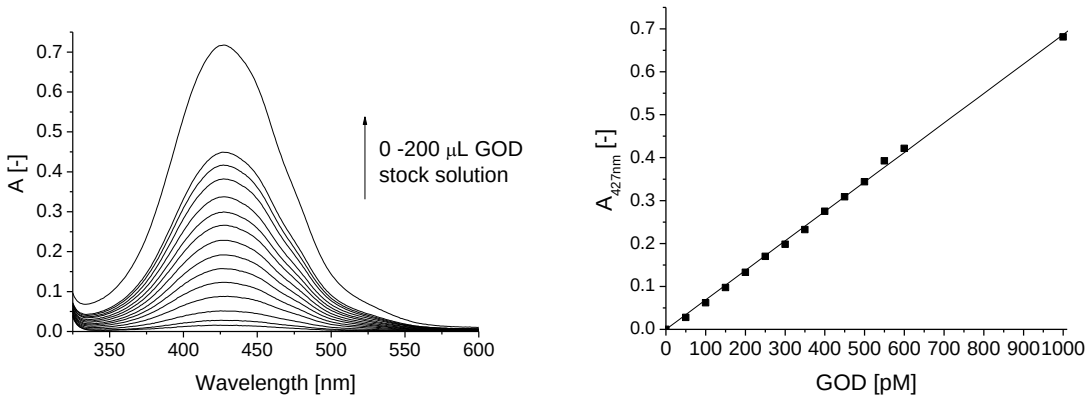


Figure S1. GOD concentration dependency of the enzymatic cascade reaction system consisting of GOD, HRP, D-glucose, and OPD. The concentrations in the solution were: 3.14 mM OPD, 3.45 mM D-glucose, and 1.98 nM HRP; pH 5.0, 10 mM phosphate, 0.15 M NaCl. The concentration of GOD was varied between 0 and 1000 pM. **(a)** Absorbance spectra of the solutions measured after 30 min incubation in the dark at room temperature. **(b)** Absorbance at 427 nm plotted *versus* GOD concentration. The r value was 0.9994.

3. Quantification of active immobilized GOD

For estimating the amount of *active* immobilized B-GOD from activity measurements, the following assumptions were made: (i) the microenvironment around the immobilized enzyme (local pH, salt concentration, presence of charged amino groups) does not influence the activity of B-GOD, *i. e.* the local enzyme surface environment is assumed to be similar to the environment of the enzyme in solution; (ii) the diffusions of the substrates D-glucose, O_2, OPD, and HRP close to the surface are the same as in solution; and (iii) the enzyme immobilized on the surface has the same catalytic efficiency as the free enzyme in solution. Then, using an appropriate calibration for GOD dissolved in solution (see Figure S1), the amount of active B-GOD molecules immobilized on the surface was calculated. In this way 0.51 pmol B-GOD per cm^2 were calculated to be bound to the surface of the sensor chip.

4. Transformation of secondary fringe patterns into absorption spectra

The secondary fringe patterns measured with the TInAS are intensity spectra and can be converted into absorption spectra, since the negative logarithm of the intensity at wavelength λ is the absorbance at λ, A_λ, (Equation S4).

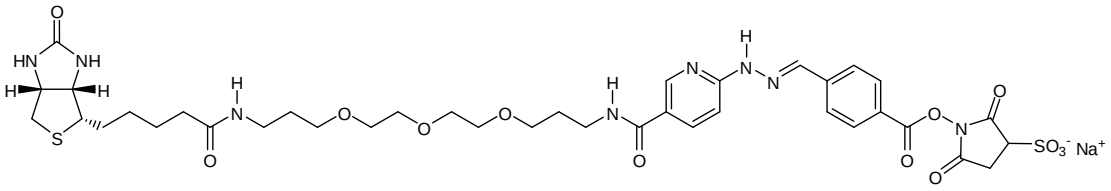
$$A_\lambda = -\log I_\lambda \qquad \text{(S4)}$$

The changes in absorbance at λ, ΔA_λ, can be measured as a function of time, t, by taking into account the intensity at λ at time t and at time 0, $I_\lambda(t)$ and $I_\lambda(0)$ respectively (Equation S5).

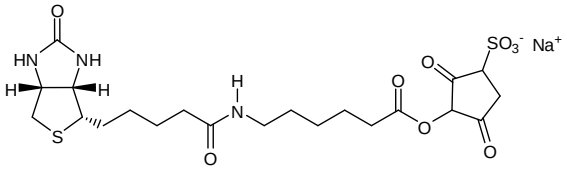
$$\Delta A_\lambda(t) = -\log\left(\frac{I_\lambda(t)}{I_\lambda(0)}\right) \qquad \text{(S5)}$$

This calculation was done for the secondary fringe patterns measured after injection of 0.5 mL of a OPD/D-glucose/HRP solution into the GOD coated TInAS flow cell (3.14 mM OPD, 3.45 mM D-glucose, 1.98 nM HRP, phosphate solution pH 5.0, 0.15 M NaCl, flow rate 2020 µl/min, t = 330 min in Figure 2). The secondary fringe patterns that were transformed into absorption spectra which are shown in Figure 3. The maximum of the absorption peak of the produced DAP is at 427 nm, in agreement with λ_{max} for the reaction carried out with both GOD and HRP dissolved in solution at pH 5.0 (Figure S1).

5. Chemical structures of ChromaLink Biotin 354 and Sulfo-NHS-LC-Biotin



ChromaLink Biotin 354 (from SoluLink)



Sulfo-NHS-LC-Biotin (from Pierce)

Scheme S1. Chemical structures of the N-hydroxysuccinimide (NHS) esters used for the biotinylation of *de*-PG2 (ChromaLink Biotin 354) and the enzymes (Sulfo-NHS-LC-Biotin).

6. Lactose detection with a three-enzyme cascade reaction system immobilized inside glass tubes (β-Gal, GOD, HRP)

6. 1. Experimental details not described in the main part of the paper

Materials: E. coli β-galactosidase (β-Gal) was from Toyobo Co. Ltd., Japan (product GLO-2022), obtained through Sorachim SA, Switzerland. The concentration of β-Gal in aqueous solutions was determined by UV/Vis spectroscopy by taking into account ε_{280nm} = 1,130,000 $M^{-1}cm^{-1}$.[6] $MgCl_2$ was from Fluka, Switzerland.

Biotinylation of β-Gal: 1 mL of a 3.4 μM β-Gal solution (prepared in 50 mM sodium phosphate, pH 7.0, 2 mM $MgCl_2$) and 30 μL of a Sulfo-NHS-LC-Biotin solution (5 μM in water) were mixed and the mixture was stirred for one hour at 500 rpm at room temperature. The reaction solution was then added onto a Sephadex G75 column (length: 30 cm; diameter: 1.2 cm; equilibrated and eluted with 10 mM phosphate solution, 0.15 M NaCl, pH 5.0; 0.5 mL/min). The fractions containing the enzyme were pooled and concentrated by ultrafiltration (Millipore 50 kDa, 20 min, 2000 rpm). The average number of biotin molecules bound per enzyme was determined with the HABA test.[3] Biotinylated β-galactosidase (B-β-Gal) was stored at 5 °C in phosphate solution at pH 7.0 (50 mM phosphate) containing 2 mM $MgCl_2$.

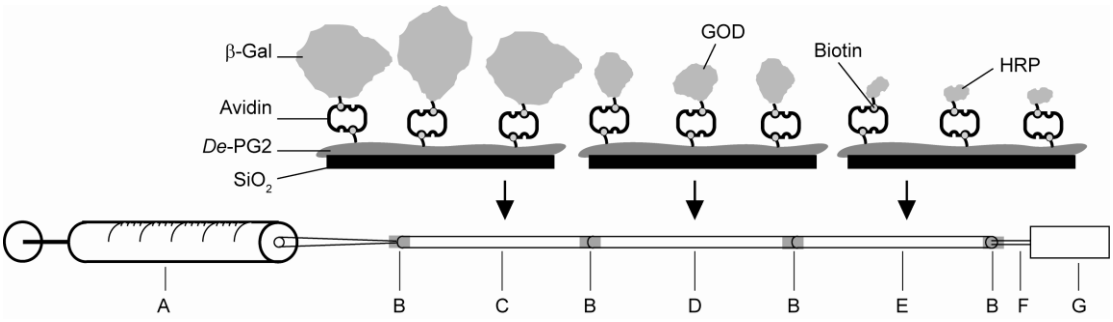
Immobilization of B-β-Gal inside glass tubes. The protocol for B-β-Gal immobilization was first studied with the TInAS and then applied to glass tubes. The procedure was optimized to achieve at most 8% unspecific binding. The first two adsorption steps (B-*de*-PG2 and avidin) were identical as described for B-HRP and B-GOD. *i.e.* first B-*de*-PG2 was adsorbed on glass (0.05 mg/mL in phosphate solution, pH 7.2, 0.15 M NaCl, incubation time 60 min), and then, after rinsing with phosphate solution (pH 7.2, 0.15 M NaCl, ~5 mL), avidin was adsorbed (0.1 mg/mL in phosphate solution, pH 7.2, 0.15 M NaCl; incubation time 60 min). Then the tubes were washed with two cycles of 10 mM phosphate solution – first pH 7.2, 0.15 M NaCl first and then pH 5.0, 1 M NaCl – and B-β-Gal was adsorbed (0.08 mg/mL in phosphate solution, pH 5.0, 1 M NaCl, incubation time 15 min). Eventually the tubes were washed with 5 mL phosphate solution 1 M NaCl, pH 5.0. After enzyme immobilization, the glass tubes were stored at 5 °C in phosphate solution (50 mM phosphate), 2 mM $MgCl_2$, pH 7.0.

Lactose quantification via the β-Gal/GOD/HRP cascade reaction inside glass tubes: Lactose was quantified *via* the three-enzyme cascade reaction using *o*-

phenylenediamine (OPD) as HRP substrate, yielding 2,3-diaminophenazine (DAP) as reaction product. First, three tubes containing in the first tube immobilized B-β-Gal, in the second immobilized B-GOD, and in the third tube immobilized B-HRP were prepared, as described in the main text and above. The three tubes were connected with silicone tubing in such a way that no visible gaps were present between the tubes. To the inlet of the first tube, a syringe pump (NE-1000 from New Era Pump System Inc., USA) was connected. The outlet of the third tube was connected to a Z-flow cell (w/SMA 905 10 mm path, microliter volume, Teflon, from Ocean Optics, USA). Figure S2a is a schematic representation of the set up. The relevant reactions involved are shown in Figure S2b. The hydrolysis of lactose is catalyzed by the immobilized B-β-Gal inside the first tube, yielding D-galactose and D-glucose. The β-form of D-glucose is then oxidized by the the immobilized B-GOD to glucono-δ-lactone and hydrogen peroxide (H_2O_2) in the presence of dissolved oxygen inside the second tube. The formed H_2O_2 activates the immobilized B-HRP inside the third tube which in turn catalyzes the oxidation of OPD present in the solution to yield DAP. The absorption spectrum of the solution eluting from the tubes was recorded between 380 and 600 nm as a function of time with a Perkin Elmer Lambda 20 spectrophotometer in which the cell holder was replaced and coupled to a fibre optics system (SMA-SMA patchcord ASB600-660UVPIT from FiberTech Optica, Canada).

The syringe was filled with an aqueous solution containing 3.14 mM OPD and up to 6.52 mM lactose in 10 mM phosphate solution, pH 7.0, and the solution was pumped through the three connected tubes at a flow rate of 30 μL/min. This flow rate was chosen as compromise between reasonable equilibration time and high enough UV/Vis signal intensity.

(a)



(b)

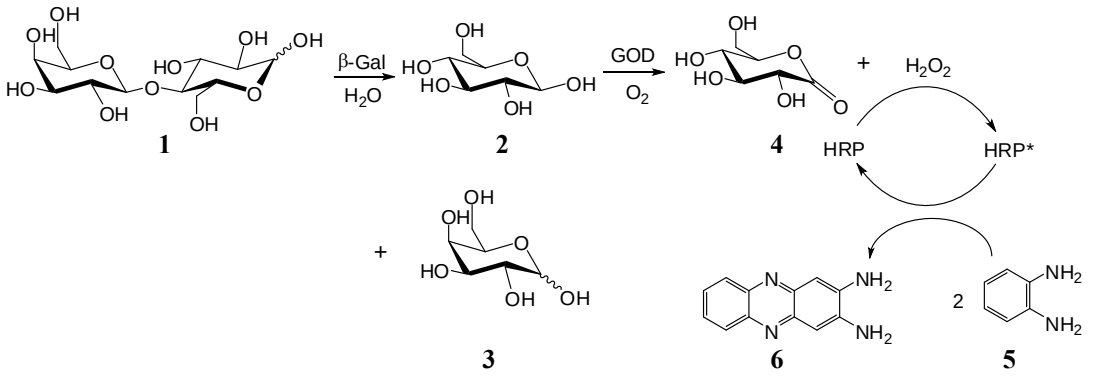


Figure S2: (a) Schematic representation of the glass tube system for the determination of lactose with immobilized B-β-Gal, B-GOD, and B-HRP. (A) Syringe containing the substrates solution: lactose (0-6.52 mM) and OPD (3.14 mM), 10 mM phosphate, 0.15 M NaCl, pH 7.0, flow rate 30 µL/min; (B) silicone tubing for connecting the syringe and the glass tubes; (C) glass tube inside which B- β-Gal was immobilized; (D) glass tubes inside which B-GOD was immobilized; (E) glass tubes inside which B-HRP was immobilized; (F) Teflon tubing connecting the output flow with the flow cell (G); (G) 10 mm flow cell connected to the UV/Vis spectrometer *via* fiber optics for detecting the amount of DAP formed (λ_{max} = 417 nm).[5,7]

(b) Enzymatic cascade reaction for the quantification of lactose. The hydrolysis of lactose (1) into D-glucose (2) and D-galactose (3) catalyzed by β-Gal. The oxidation of β-D-glucose (2) in presence of oxygen to glucono-δ-lactone (4) and hydrogen peroxide is catalyzed by GOD. Eventually, two OPD molecules (5) are oxidized to DAP (6) in presence of HRP and the formed H_2O_2.

6. 2. Preliminary results

As schematically shown in Figure S2a, three glass tubes modified with immobilized B-β-Gal inside the first tube, immobilized B-GOD in the second tube and immobilized B-HRP in the third tube were prepared and connected. An aqueous solution containing OPD (3.14 mM) and various amounts of lactose (0-6.52 mM) were pumped through the tube system and the efflux was analyzed by measuring the absorption spectrum between 380 and 600 nm as a function of time (one spectrum per minute). The recorded spectra are plotted in Figure S3a. The absorption maximum was found to be at 417 nm, in agreement with λ_{max} of DAP at pH 7.0.[7] Figure S3b is a plot of A_{417nm} *vs*. time for different lactose concentrations. After about 50 min, a constant value was reached. This steady state value of A_{417nm} measured between 55 and 65 min after the start of pumping the solution into the tubes is shown as a function of the lactose concentration in Figure S3c. The data in Figure S5c clearly show that an increase of the concentration of lactose led to an increase of the amount of DAP formed.

If one of the glass tubes containing immobilized enzymes was replaced by a glass tube which did not contain the enzyme, *i. e*. a tube containing only B-*de*-PG2 and avidin, no significant absorption was recorded ($A_{417nm} < 0.02$). Thus, all three enzymes are necessary for this lactose detection. Moreover, if the sequence of the glass tubes containing the immobilized enzymes was changed, only the background signal was measured.

6. 3. Conclusion

The concept of immobilizing enzymes inside glass tubes with the help of *de*-PG2 and the avidin/biotin system has been applied to three enzymes, the monomeric HRP, the dimeric GOD and the tetrameric β-Gal. The immobilization procedure is highly reproducible and the immobilized enzymes remain catalytically active, allowing the use of connected tubes containing the three enzymes in proper sequence for the spectrophotometric determination of lactose *via* a three-enzyme cascade reaction. The results obtained demonstrate the versatility of the method which involves the biotinylated polycationic dendronized polymer *de*-PG2 which forms an efficient soft organic layer with strong adherence to the inorganic glass surface.

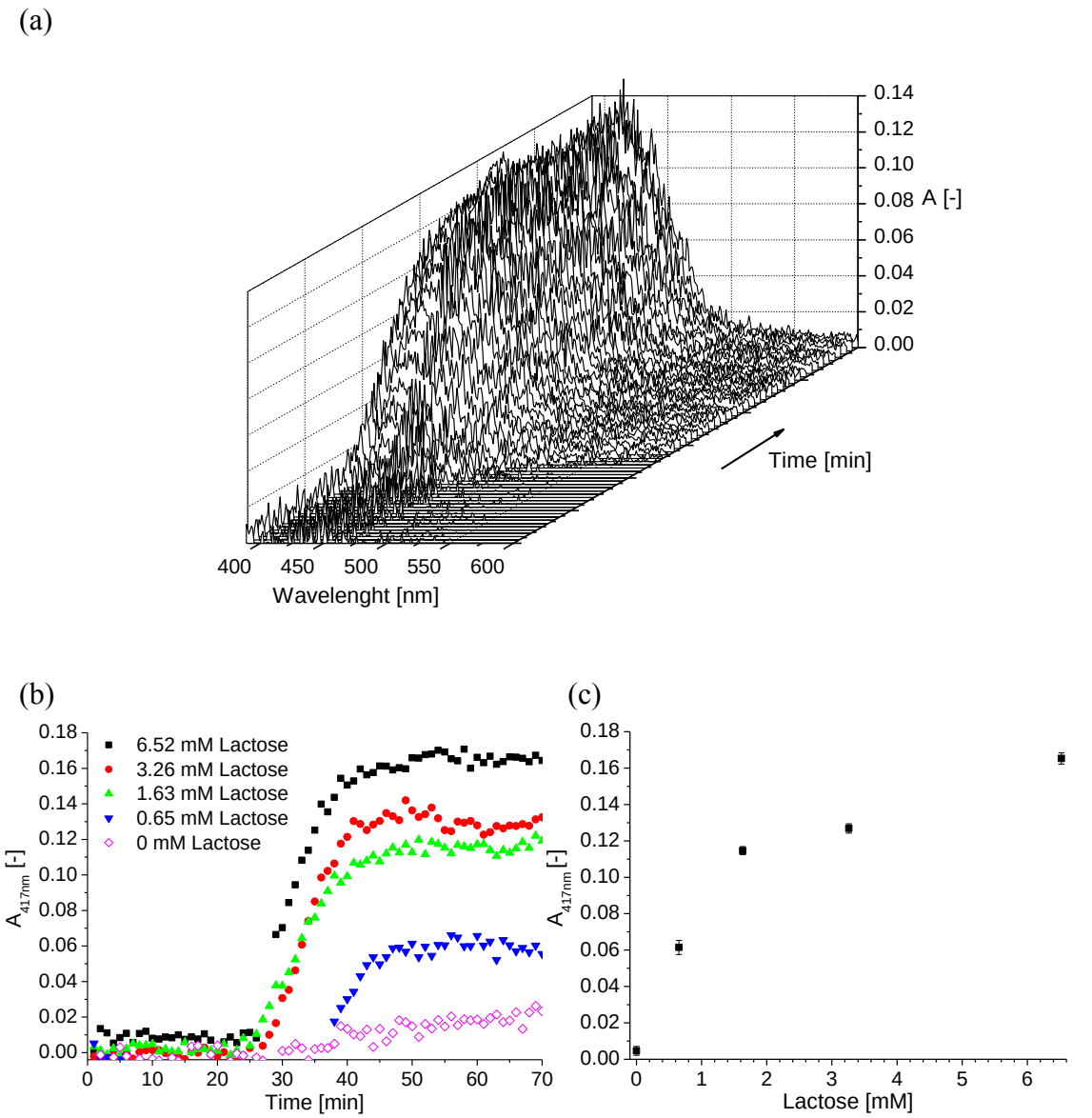


Figure S3: B-β-Gal/B-GOD/B-HRP glass tube system for the determination of lactose in aqueous solutions, see setup in Figure S4a. The OPD/lactose solutions (3.14 mM OPD, 0-6.52 mM lactose, 10 mM phosphate, 0.15 M NaCl, pH 7.0, flow 30 µL/min) were injected into the β-Gal/GOD/HRP glass tube system at t=0 and the output solution was analyzed with online UV/Vis spectroscopy, see Figure S4a. **(a)** Absorbance spectra of the output flow measured every minute for 3.26 mM lactose, 3.14 mM OPD; **(b)** Dependency of A_{417nm} (originating from the formation of DAP) on time for different lactose concentrations (0-6.52 mM); **(c)** Average and standard deviation of the A_{417nm} values measured between 55 and 65 min *versus* lactose concentration.

7. References

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