Application of an enzymatic cascade reaction for the synthesis of the emeraldine salt form of polyaniline

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Abstract

The synthesis of the emeraldine salt form of polyaniline (PANI-ES) from aniline with *Aspergillus* sp. glucose oxidase (GOD), D-glucose, dissolved O₂, and horseradish peroxidase isoenzyme C (HRPC) in the presence of large unilamellar vesicles of AOT (sodium bis-(2-ethylhexyl)sulfosuccinate) as templates at pH = 4.3 and *T* ~ 25 °C was investigated in a systematic way. In this cascade reaction mixture, the oxidation of aniline is catalyzed by HRPC with H₂O₂ that is formed in situ as byproduct of the GOD-catalyzed oxidation of D-glucose with O₂. Under the elaborated experimental conditions which we considered ideal, the formation of PANI-ES products is evident, as judged by UV/Vis/NIR and EPR measurements. Comparison was made with a reference reaction, which was run under similar conditions with added H₂O₂ instead of GOD and D-glucose. Although the reference reaction was found to be superior, with the cascade reaction, PANI-ES products can still be obtained with high aniline conversion (> 90%) within 24 h as stable dark green PANI-ES/AOT vesicle dispersion. Our results show that the in situ formation of H₂O₂ does not prevent the inactivation of HRPC known to occur in the reference reaction. Moreover, the GOD used in the cascade reaction is inactivated as well by polymerization intermediates.

Keywords Aniline · Glucose oxidase · Horseradish peroxidase · Template · Polyaniline · Vesicles

Introduction

Although there are well-established procedures for the chemical (Huang et al. 1986; Stejskal and Gilbert 2002; Surwade et al. 2009; Stejskal et al. 2015) or electrochemical (Huang et al. 1986; Okamoto and Kotaka 1998; Stejskal et al. 2015) synthesis of the electroconductive polyaniline (PANI), the potential of using oxidative enzymes as biocatalysts is also being explored (Xu et al. 2006; Bouldin et al. 2010; Cruz-Silva et al. 2011; Walde and Guo 2011; Otrokho et al. 2013; Ćirić-Marjanović et al. 2017), most often by applying a heme peroxidase with hydrogen peroxide (H₂O₂) as oxidant (Liu et al. 1999a, b; Sakharov et al.

2003; Caramyshev et al. 2005; Rumbau et al. 2007; Junker et al. 2012, 2013) or a high potential laccase in the presence of molecular oxygen (O₂) (Karamyshev et al. 2003; Shumakovich et al. 2012; Junker et al. 2014; Zhang et al. 2016; Walde et al. 2019). The enzymatic approach allows carrying out the synthesis under milder, environmentally friendlier conditions as compared to chemical methods, at least if the acidity of the reaction medium is compared: pH ≈ 3.5–4.5 in the case of the enzymes, pH < 2.5 for the chemical approach (Stejskal et al. 2015). Moreover, in the case of the enzymatic approach with H₂O₂ or O₂ as oxidant, the side product is H₂O (Junker et al. 2012, 2014), while oxidation with the most popular chemical oxidant, ammonium peroxydisulfate (APS) (Stejskal and Gilbert 2002), yields sulfuric acid as side product (Stejskal et al. 2015). Obviously, for such comparison the costs of the enzymes—as compared to APS, for example—should not be ignored (Junker et al. 2012, 2014). Only if cheap, industrial grade enzymes can be used, the enzymatic approach might compete economic-wise with the traditional chemical methods. On the other hand, the chemical structure, average molar mass, polydispersity, and “solubility” or dispersibility of the PANI products obtained may not only depend on the *synthesis method* used, but also

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on the *precise experimental conditions* at which the synthesis is carried out with one chosen method (Kashima et al. 2018). With the milder enzymatic approach, products with superior, desired properties—as compared to the chemical or electrochemical methods—might be obtained. For exploring this possibility, detailed studies with enzymes as catalysts are worth pursuing. The work we present here is one of such studies.

In terms of “desired properties,” the emeraldine salt form of polyaniline (PANI-ES) is of particular interest for many possible applications, as it is the only (or most relevant) electroconductive form of PANI (Chiang and MacDiarmid 1986; Focke and Wnek 1988; Stejskal et al. 2015; Petrova et al. 2012). In Scheme 1, the chemical structure of the repeating unit (r.u.) of an ideal PANI-ES chain is shown. It consists of four linearly connected aniline building blocks that are bonded via the nitrogen atoms and the carbon atoms in *para*-position to the amino group. Moreover, two of the four aniline building blocks in the PANI-ES r.u. are oxidized and protonated and two are reduced (and not protonated). This half-oxidation state of the r.u. may result in the formation of the polaron forms of PANI-ES with two unpaired electrons per r.u. and all four benzene rings with fully delocalized π electrons (benzenoid structure). One usually distinguishes polaron pairs from separated polarons (Scheme 1) (Wallace et al. 2009; Dmitrieva and Dunsch 2011). In the bipolaron form of the PANI-ES r.u., unpaired electrons do not exist (benzenoid and quinoid structures). From many previous studies, it is known that the nature of the counter ion (“dopant” A^- in Scheme 1) has an influence on the conductivity of PANI-ES in the solid state (Stejskal et al. 2015).

PANI-ES has characteristic absorptions in the near-infrared (NIR) region, with maximal absorbance at $\lambda_{\max} \geq 800$ nm (previously assigned to the $\pi \rightarrow$ polaron transition), at $\lambda_{\max} \approx 420$ nm (polaron $\rightarrow \pi^*$ transition) and at $\lambda_{\max} \approx 300$ nm ($\pi \rightarrow \pi^*$ transition) (Huang and MacDiarmid 1993; Nekrasov et al. 2001; do Nascimento and de Souza 2010; Bilal et al. 2015). Furthermore, low absorbance at $\lambda = 500\text{--}600$ nm is indicative for a low extent of chain branching (Liu et al. 1999b) or low extent of phenazine formation (Luginbühl et al. 2017). Recently, the above widely accepted assignments of the electron transitions at $\lambda \geq 800$ nm and $\lambda \approx 420$ nm to polaron transitions (Huang and MacDiarmid, 1993) have been questioned (Lin et al. 2017; Mills et al. 2019). It is the observed low EPR signal intensity of PANI-ES-like oligomers that led to the suggestion that the strong absorption band in the near-infrared region of the spectrum might actually originate from transitions involving bipolarons and not polarons (Lin et al. 2017; Mills et al. 2019). Nevertheless, and independent from the correct NIR absorption band assignment, the mentioned characteristic spectroscopic features are typical for conductive PANI-ES, and they are often taken as a measure for optimizing reaction

Scheme 1. Chemical structures of the bipolaron and the two polaron forms of the repeating unit (r.u.) of PANI-ES, the conductive form of polyaniline; A^- represents the counter ion (dopant) (Chiang and MacDiarmid 1986; Wallace et al. 2009; Dmitrieva and Dunsch, 2011). The structures shown refer to ideal PANI-ES chains which are thought to consist of polaron or bipolaron segments only

conditions in cases where the reaction products do not precipitate but remain dissolved or dispersed in solution and therefore can be analyzed by in situ UV/Vis/NIR absorption measurements. This was the case in several studies carried out in the past on the peroxidase/ H_2O_2 - or laccase/ O_2 -catalyzed oxidation of aniline in the presence of so-called “templates” (Liu et al. 1999a; Walde and Guo 2011; Junker et al. 2012).

“Templates” are additives which (i) influence the oxidation and polymerization reaction in a positive way, (ii) act as counter ions and (iii) help keeping the formed products dissolved or dispersed (Liu et al. 1999a; Walde and Guo

2011; Serrano-Luginbühl et al. 2018). Good templates for obtaining PANI-ES enzymatically from aniline, or the aniline dimer PADPA (*p*-aminodiphenylamine), were found to be anionic polyelectrolytes like sulfonated polystyrene sodium salt (SPS) (Samuelson et al. 1998; Liu et al. 1999b; Karamyshev et al. 2003; Sakharov et al. 2003; Fujisaki et al. 2019; Kashima et al. 2019), or anionic micelles, formed for example from SDBS (sodium dodecylbenzenesulfonate) (Liu et al. 1999a, 2002; Streltsov et al. 2009; Fujisaki et al. 2019; Kashima et al. 2019), or anionic vesicles, built from sodium bis(2-ethylhexyl)sulfosuccinate) (AOT) (Guo et al. 2011; Junker et al. 2012, 2014; Fujisaki et al. 2019; Kashima et al. 2019) or from a 1:1 molar mixture of SDBS and deca-noic acid (Guo et al. 2009; Fujisaki et al. 2019; Kashima et al. 2019).

Toward possible applications of enzymatically prepared polyaniline, in a recent study it was shown that PANI-ES obtained from aniline in the presence of AOT vesicles with horseradish peroxidase (HRP) and H_2O_2 might by suitable as photothermal agent for tumor theranostics (Zhang et al. 2020). In another area of research, an artificial cell-like reproduction system is being developed, which is based on the AOT vesicle-guided, HRP/ H_2O_2 -catalyzed synthesis of PANI-ES products (Kurusu et al. 2019). In that work, chemistry and soft matter physics are linked. More specifically, micrometer-sized giant AOT vesicle membranes work as templates for the synthesis of PANI-ES products, and the PANI-ES products simultaneously work as templates for the incorporation of AOT molecules from the bulk solution into the AOT membranes, which leads to vesicle membrane growth. Based on such mutual catalytic coupling between AOT membrane growth and PANI-ES product formation, AOT-based vesicles not only show growth in size but they also divide, *i.e.*, vesicle reproduction occurs (Kurusu et al. 2019). In that work, the main focus is not on the properties of the PANI-ES products obtained, but on the physical analysis of changes of vesicle size and morphology in a constant chemical environment: AOT and H_2O_2 molecules are continuously supplied to a giant target vesicle which is built of mainly AOT molecules.

In all these examples, the terminal oxidant, H_2O_2 or O_2 , was added at—or was present from—the beginning of the reaction (Guo et al. 2011; Junker et al. 2012; Fujisaki et al. 2019; Kashima et al. 2019), or it was added in portions during the course of the reaction (H_2O_2) (Samuelson et al. 1998; Liu et al. 1999a, b, 2002; Sakharov et al. 2003). The aim of the work presented here was to investigate whether PANI-ES formation from aniline with horseradish peroxidase isoenzyme C (HRPC) and AOT vesicles as templates is also possible with in situ formation of H_2O_2 from D-glucose with glucose oxidase (GOD) and O_2 from air according to the following equation:

$\beta\text{-D-glucose} + \text{O}_2 \rightarrow \text{D-glucono-}\delta\text{-lactone} + \text{H}_2\text{O}_2$ (Bankar et al. 2009), see Scheme 2. Since such GOD-catalyzed in situ formation of H_2O_2 in the presence of HRPC and aniline resulted in the formation of PANI-ES products, the two questions we wished to answer were the following. (a) How do the progress of the reaction and the spectroscopic properties of the obtained PANI-ES products compare with those of the PANI-ES products obtained under similar conditions without GOD and D-glucose but addition of H_2O_2 at the beginning of the reaction? (Junker et al. 2012) And (b) Is there an advantage of using the enzymatic cascade reaction involving GOD and HRPC over the use of a single enzyme (HRPC), for example in terms of HRPC inactivation caused by H_2O_2 ? The inactivation of heme peroxidases by high amounts of H_2O_2 is well known since many years (Arnao et al. 1990; Hiner et al. 1996; Mao et al. 2014). With a controlled enzymatic in situ formation of H_2O_2 , exposure of HRPC to high H_2O_2 concentrations can be avoided or minimized.

In a broader context, enzymatic cascade reactions not only are essential for the efficient transformation of metabolites in each living cell, but they are also of great interest for the sustainable industrial synthesis of organic compounds (Wohlgemuth 2010; Ricca et al. 2011; Choi et al. 2015; Sigrist et al. 2015; Schmidt-Dannert and Lopez-Gallego 2016).

Experimental

Materials

Aniline (> 99%), hydrogen peroxide (30% in water, ~9.8 M), sodium dihydrogenphosphate (NaH_2PO_4) dihydrate (> 99%), and D(+)-glucose (> 99%), chloroform (CHCl_3 , > 99%), acetonitrile (CH_3CN , > 98%) and perchloric acid (HClO_4 , 60% in water) were purchased from Wako Pure Chemical Industries (Japan). AOT (sodium bis-(2-ethylhexyl) sulfosuccinate, > 99%) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($\text{ABTS}^{2-}(\text{NH}_4^+)_2$, > 98%) were purchased from Sigma-Aldrich. Oxo[5,10,15,20-tetra(4-pyridyl)porphyrinato] titanium (IV) (> 90.0%) was purchased from Tokyo Chemical Industry (Japan). Horseradish peroxidase isoenzyme C (HRPC, Grade I, PEO-131, 286 U/mg, RZ = 3.13, $M \sim 40$ kDa, Lot No. 74590) and Glucose oxidase from *Aspergillus* sp. (GOD, Grade II, GLO-201, 166 U/mg, SA = 205, $M \sim 153$ kDa, Lot No. 74180) were purchased from Toyobo Enzymes (Japan). All other chemicals used were of research grade. A 100 mM dihydrogen phosphate solution was prepared by using NaH_2PO_4 , deionized water and small amounts of H_3PO_4 to obtain pH = 4.3. This solution is called “100 mM NaH_2PO_4 solution (pH = 4.3)”.

Scheme 2. Illustration of the two-enzyme cascade reaction used for the oxidation of aniline monomers to anilino radicals that undergo follow-up reactions (Junker et al. 2012). The two reaction steps are (1) the formation of H₂O₂ (3) and D-glucono-δ-lactone (4) from D-glucose (1) and dissolved O₂ (2), whereby 4 is hydrolyzed to D-gluconic acid (5). In step (2), 3 oxidizes the heme enzyme HRPC in a two electron oxidation reaction to [HRPC-Fe(IV)=O]^{•+} (the so-called compound I, with a radical cation localized on the porphyrin ring), followed by two one-electron oxidations of two molecules of

aniline (6) to two anilino radicals (7), via compound II (not shown) (Junker et al. 2012, 2013). Oligomeric and polymeric products are obtained from 7, 3, and 6, most likely without direct involvement of the enzymes (Junker et al. 2012). Not shown is the role "template" molecules play that are present in the reaction mixture (AOT in the present work): (i) for guiding the reactions, (ii) for acting as counter ions, and (iii) for preventing product precipitation. FAD stands for flavin adenine dinucleotide, the cofactor of GOD

AOT vesicle preparation and CVC determination

AOT vesicle dispersions were prepared as described before with the polycarbonate membrane extrusion method (Junker et al. 2012). Solid AOT (89.0 mg) was added to a 250-mL round bottom flask and then dissolved in 5 mL chloroform, followed by the formation of a thin AOT film upon removal of chloroform with a rotary evaporator. For complete chloroform removal, the AOT film was put under high vacuum overnight. The AOT film was then hydrated and dispersed by using 10 mL of a 100 mM NaH₂PO₄ solution (pH=4.3) or of a 100 mM NaH₂PO₄ solution containing a defined concentration of D-glucose (pH=4.3). For most of the experiments, [D-glucose] = 100 mM (see below). The obtained 20 mM AOT vesicle dispersions were frozen by placing the round bottom flask in liquid nitrogen, followed by thawing in a water bath heated to 60 °C. This procedure was repeated ten times. The vesicle dispersions were first extruded ten times through 200-nm-pore-size nucleopore polycarbonate

membranes, and then, another ten times through 100-nm pore-size membranes, using "The Extruder" from Lipex Biomembranes (Vancouver, Canada). The resulting AOT vesicle dispersions mainly contained large unilamellar vesicles (LUVs) with an average diameter of about 80–100 nm (Guo et al. 2011; Junker et al. 2012). The vesicle dispersions were stored at room temperature (*T* ~ 25 °C) and used within 14 days after preparation.

The critical concentration for vesicle formation (CVC) of AOT in 100 mM NaH₂PO₄ solution (pH=4.3) and in 100 mM NaH₂PO₄/100 mM D-glucose solution (pH=4.3) was estimated by turbidity measurement, as described before (Guo et al. 2011). A 20 mM AOT LUV dispersion that was extruded five times through 200-nm-pore-size nucleopore polycarbonate membranes was first diluted with 100 mM NaH₂PO₄ solution (pH=4.3) to different desired AOT concentrations. After incubation for 24 h at *T* ~ 25 °C, the turbidity determined as absorbance at the arbitrarily chosen wavelength *λ* = 400 nm, *A*₄₀₀, was measured for each

diluted sample by using a V-670 UV/Vis/NIR spectrophotometer from JASCO (Japan), and quartz cells with optical path lengths of $L = 1$ mm. A_{400} values were plotted vs. AOT concentration. The CVC value was taken as the lowest AOT concentration at which A_{400} clearly deviated from 0 (~ 0.4 mM), see Fig. S-1. This value is in agreement with previous determinations (Guo et al. 2011).

“Reference reaction”: synthesis of PANI-ES products from aniline with HRPC, H_2O_2 , and AOT vesicles

The conditions for the “reference reaction” were the ones elaborated before (Junker et al. 2012) and already used in various previous investigations (Junker et al. 2013; Pašti et al. 2017; Fujisaki et al. 2019). The reactions were carried out in 2-mL polypropylene Eppendorf tubes. All components of the reaction mixture, except H_2O_2 , were added to 350 μ L NaH_2PO_4 solution (100 mM, pH = 4.3): 75 μ L AOT LUV dispersion (20 mM in 100 mM NaH_2PO_4 solution), 50 μ L aniline solution (40 mM in 100 mM NaH_2PO_4 solution, pH adjusted to 4.3 with H_3PO_4) and 25 μ L HRPC solution (18.4 μ M HRPC in 100 mM NaH_2PO_4 solution, pH = 4.3, spectrophotometrically determined using the known molar absorptivity at $\lambda = 403$ nm, $\epsilon_{403}(\text{HRPC}) = 1.02 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (Dunford and Stillman 1976). After gentle mixing, the reaction was triggered by quick addition of 1.13 μ L H_2O_2 solutions (2.0 M in water) and further gentle mixing. The initial reaction conditions were as follows: 3.0 mM AOT, 4.0 mM aniline, 0.92 μ M HRPC and 4.5 mM H_2O_2 , in 100 mM NaH_2PO_4 solution (pH = 4.3), reaction volume = 0.50 mL, $T = 25^\circ\text{C}$, reaction time $t = 24$ h. The reaction tubes were used with closed caps and kept standing during the reaction (no tube rotation), unless indicated otherwise.

“Cascade reaction”: synthesis of PANI-ES products from aniline with D-glucose, GOD, dissolved O_2 , HPPC, and AOT vesicles

The conditions for the “cascade reaction” run in 5-mL polypropylene Eppendorf tubes were the following. All components of the reaction mixture, except the GOD solution, were added to 300 μ L of 100 mM NaH_2PO_4 solution (pH = 4.3) containing defined concentrations of D-glucose: 75 μ L AOT LUV dispersion (20 mM in 100 mM NaH_2PO_4 solution containing a defined concentration of D-glucose), 50 μ L aniline solution (40 mM in 100 mM NaH_2PO_4 solution, pH adjusted to 4.3 with H_3PO_4) and 25 μ L HRPC solution (18.4 μ M, prepared in 100 mM NaH_2PO_4 solution, pH = 4.3, and spectrophotometrically determined, see above). After gentle mixing, the reaction was triggered by quick addition of 50 μ L of a GOD solution of defined GOD concentration (prepared in 100 mM NaH_2PO_4 solution,

pH = 4.3, spectrophotometrically determined by using $\epsilon_{450}(\text{GOD}) = 2.82 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) (Swoboda and Massey 1965), followed by gentle mixing and closure of the caps of the reaction tubes and sealing with Parafilm (to avoid leakage of solution and air exchange). The tubes were then placed in the holes of a home-made rotary mixing device and continuously rotated at ~ 90 rpm (see Fig. S-2) during the reaction, unless indicated otherwise. The initial reaction conditions were as follows: 3.0 mM AOT, 4.0 mM aniline, 0.92 μ M HRPC and defined concentrations of GOD (0.2 μ M for the “ideal conditions,” see below), and D-glucose (100 mM for the “ideal conditions,” see below), in 100 mM NaH_2PO_4 solution (pH = 4.3), reaction volume = 0.50 mL, $T = 25^\circ\text{C}$, $t = 24$ h. The desired D-glucose concentration in the pre-laid 300 μ L D-glucose solution was obtained by mixing defined volumes of a D-glucose stock solution of either 250 mM or 1 M (prepared in 100 mM NaH_2PO_4 , pH = 4.3) and an appropriate volume of 100 mM NaH_2PO_4 solution (pH = 4.3). The D-glucose stock solutions were prepared at least 24 h before use to allow D-glucose to reach equilibrium between the α - and β -forms.

UV/Vis/NIR and EPR absorption measurements

All UV/Vis/NIR absorption measurements were carried out with a V-670 UV/Vis/NIR spectrophotometer from JASCO (Japan) using quartz cuvettes of either $L = 1$ mm or 10 mm optical path length (Junker et al. 2012). Electron paramagnetic resonance (EPR) measurements of the reaction mixtures were measured with a Bruker EMX X-band spectrometer equipped with a TM cavity. The spectra were measured at X-band microwave frequency with a modulation frequency of 100 kHz and modulation amplitudes of 1 G at room temperature (Junker et al. 2012).

Quantification of aniline and H_2O_2 in the “cascade reaction mixtures” during the reaction

The amounts of remaining aniline and formed H_2O_2 in the “cascade reaction mixtures” were determined by using the same methods as described before (Junker et al. 2012). For the quantification of remaining amounts of aniline, 30 μ L of the reaction mixture was added to 1470 μ L acetonitrile inside a 2-mL polypropylene Eppendorf tube. After centrifugation, the UV/Vis/NIR absorption spectrum of the supernatant solution was recorded. From the absorption intensity at $\lambda = 238$ nm, the concentration of aniline was calculated based on $\epsilon_{238}(\text{aniline}) = 1.01 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, which was determined from a calibration curve obtained with known amounts of aniline (Fig. S-3). For the quantification of H_2O_2 , a spectrophotometric method was used that is based on the reaction between added oxo[5,10,15,20-tetra(4-pyridyl)porphyrinato]titanium(IV)—Ti-TPyP—and H_2O_2 present

in the "cascade reaction mixture" (Takamura and Matsumoto 2009). The following procedure was used: 125 μL of diluted or undiluted reaction mixture was added to 125 μL of a 4.8 M perchloric acid solution placed inside. Afterwards, 125 μL of a Ti-TPyP solution (50 μM in 50 mM HCl) was quickly added. After gentle mixing and standing for 5 min at $T \sim 25^\circ\text{C}$, 875 μL of deionized water was added and the solution was centrifuged. The absorption intensity at $\lambda = 432\text{ nm}$ of the supernatant solution, A_{432} , was measured, then this value was compared to a blank sample which did not contain H_2O_2 . The concentration of H_2O_2 was determined from a calibration curve obtained with known amounts of H_2O_2 , plotted against $\Delta A_{432} = A_{432}(\text{blank}) - A_{432}(\text{sample})$. See Fig. S-4.

Determination of the HRPC activity in the "cascade reaction mixtures" during the reaction

The activity of HRPC in samples withdrawn from the "cascade reaction mixtures" was determined spectrophotometrically with ABTS^{2-} as reducing substrate and H_2O_2 as oxidant in 10 mM MOPS buffer solution ($\text{pH} = 7.0$) according to the protocol described by Ghéczy et al. (2016) (Ghéczy et al. 2016). The following solutions were added in the sequence given to a 1 mm quartz cuvette at $T \sim 25^\circ\text{C}$, followed by measuring the linear increase in A_{414} during the first 60 s (formation of $\text{ABTS}^{\bullet-}$): 960 μL MOPS buffer solution (10 mM, $\text{pH} = 7.0$), 10 μL reaction mixture containing HRPC, 20 μL ABTS^{2-} stock solution (50 mM in MOPS buffer solution, $\text{pH} = 7.0$), and finally 10 μL H_2O_2 stock solution (20 mM in deionized water). After gentle mixing, the slope $\Delta A_{414}/\Delta t$ was taken as measure for the activity of HRPC. The total assay volume was 1.0 mL and the initial substrate concentrations were $[\text{ABTS}^{2-}]_0 = 1.0\text{ mM}$ and $[\text{H}_2\text{O}_2]_0 = 0.2\text{ mM}$. A calibration curve made with known amounts of HRPC is shown in Fig. S-5A. The same conditions were also used for determining the activity of HRPC (0.92 μM) during storage (no "cascade reaction"), at $T \sim 25^\circ\text{C}$ for $t = 24\text{ h}$ in 100 mM NaH_2PO_4 solution, $\text{pH} = 4.3$, in the absence or presence of AOT vesicles (3 mM AOT). Under both conditions, the activity of HRPC remained constant, in agreement with previous findings (Junker et al. 2012), see Fig. S-5B.

Determination of the GOD activity in the "cascade reaction mixtures" during the reaction

The activity of GOD in samples withdrawn from the "cascade reaction mixtures" was determined spectrophotometrically with D-glucose and ABTS^{2-} in 10 mM MOPS buffer ($\text{pH} = 7.0$) according to the protocol described before (Ghéczy et al. 2016), with slight modifications.

The following solutions were added in the sequence given to a 1 mm quartz cuvette at $T \sim 25^\circ\text{C}$, followed by measuring the linear increase in A_{414} during the first 180 s (formation of $\text{ABTS}^{\bullet-}$): 865 μL MOPS buffer solution (10 mM, $\text{pH} = 7.0$), 10 μL reaction mixture containing GOD, 5 μL HRPC stock solution (20 μM in MOPS buffer solution, $\text{pH} = 7.0$), 20 μL ABTS^{2-} stock solution (50 mM in MOPS buffer solution, $\text{pH} = 7.0$), and finally 100 μL D-glucose stock solution (1.0 M in MOPS buffer solution, $\text{pH} = 7.0$). After gentle mixing, the slope $\Delta A_{414}/\Delta t$ was taken as measure for the activity of GOD. The total assay volume was 1.0 mL, $[\text{HRPC}] = 0.1\text{ }\mu\text{M}$, and the initial substrate concentrations were $[\text{D-glucose}]_0 = 100\text{ mM}$, and $[\text{ABTS}^{2-}]_0 = 1.0\text{ mM}$. A calibration curve made with known amounts of GOD is shown in Fig. S-6A. The same conditions were also used for determining the activity of GOD (0.2 μM) during storage (no "cascade reaction"), at $T \sim 25^\circ\text{C}$ for $t = 24\text{ h}$ in 100 mM NaH_2PO_4 solution, $\text{pH} = 4.3$, in the absence or presence of AOT vesicles (3 mM AOT). Under both conditions, the activity of GOD remained constant, see Fig. S-6B.

Quantification of D-glucose in the "cascade reaction mixtures" during the reaction

Remaining D-glucose in the "cascade reaction mixtures" was determined spectrophotometrically by withdrawing small volumes from the "cascade reaction mixtures" and then using the GOD/HRP/ ABTS^{2-} assay, as described above. To eliminate GOD and HRPC present in the "cascade reaction mixtures", the two enzymes were first separated from the reaction mixtures by ultrafiltration using Amicon® Ultra 0.5-mL centrifugal filters (regenerated cellulose, nominal molecular weight limit of 10 kDa) from Merck (Germany). The purified, enzyme-free D-glucose solutions were then analyzed for D-glucose content by adding the following solutions in the sequence given to a 1-mm quartz cuvette at $T \sim 25^\circ\text{C}$, followed by measuring the linear increase in A_{414} during the first 180 s (formation of $\text{ABTS}^{\bullet-}$): 945 μL MOPS buffer solution (10 mM, $\text{pH} = 7.0$), 20 μL GOD stock solution (50 nM in 10 mM MOPS buffer solution, $\text{pH} = 7.0$), 5 μL HRPC stock solution (20 μM in MOPS buffer solution, $\text{pH} = 7.0$), 20 μL ABTS^{2-} stock solution (50 mM in MOPS buffer solution, $\text{pH} = 7.0$), and finally 20 μL of the purified, enzyme-free D-glucose solution. After gentle mixing, the slope $\Delta A_{414}/\Delta t$ was taken as measure for the amount of D-glucose present in the assay mixture. The total assay volume was 1.0 mL, $[\text{GOD}] = 1\text{ nM}$, $[\text{HRPC}] = 0.1\text{ }\mu\text{M}$, and $[\text{ABTS}^{2-}]_0 = 1.0\text{ mM}$. A calibration curve made with known amounts of D-glucose is shown in Fig. S-7.

Results and discussion

Synthesis of PANI-ES from aniline with HRPC and H_2O_2 in the presence of AOT vesicles ("reference reaction")

From our previous investigations with HRPC and H_2O_2 , the following initial conditions turned out to be ideal for obtaining PANI-ES products in the presence of AOT vesicles as templates in aqueous solution (Junker et al. 2012): 3.0 mM AOT, 4.0 mM aniline, 0.92 μM HRPC, 4.5 mM H_2O_2 , pH = 4.3 (100 mM NaH_2PO_4), $T = 25^\circ\text{C}$, $t = 24$ h. With these conditions of our "reference reaction", (i) the initially added aniline is oxidized to about 90–95% (aniline conversion, *i.e.*, only 5–10% of the added aniline remain at the end of the reaction), (ii) the initially colorless reaction mixture becomes first blue and finally dark green with absorption maxima at $\lambda \approx 1000$, 420, and 300 nm, and (iii) the absorption at $\lambda \approx 500$ nm is relatively low (Fig. S-8). These phenomena are characteristic for the formation of PANI-ES, see the Introduction. The formation of products that are rich in PANI-ES r.u. is further supported by the fact that the reaction products produce an EPR spectrum (indication of the presence of unpaired electrons, as expected for the polaron form of PANI-ES) (Junker et al. 2012; Fujisaki et al. 2019). Moreover, the PANI-ES products remain dispersed (no precipitation), and the chosen aniline concentration is such that the progress of the reaction can easily be followed by in situ UV/Vis/NIR absorption measurements using conventional quartz cells with an optical path length of $L = 0.1$ cm (Junker et al. 2012). We call the conditions mentioned above "optimal" for the "reference reaction" since they fulfill several criteria we set for the reaction course and results (Junker et al. 2012; Fujisaki et al. 2019). The "reference reaction" is usually carried out in closed reaction tubes without any stirring or tube rotation. As shown in Fig. S-9, continuous rotation of the reaction tube during the reaction has no influence on the UV/Vis/NIR absorption spectrum after $t = 24$ h, *i.e.*, it has no significant influence on the outcome of the "reference reaction." The only clear drawback of the reaction is the high amount of HRPC required for high aniline conversion (0.92 μM for 4.0 mM aniline) (Junker et al. 2012). Nevertheless, the electrochemical properties of the obtained PANI-ES products are very promising (easy coating of glassy carbon electrodes and excellent capacitive properties) (Pašti et al. 2017).

For the aim of the present work, we replaced the initial addition of H_2O_2 with a mixture of D-glucose and GOD (and dissolved O_2), but kept all other conditions the same.

Fig. 1 UV/Vis/NIR absorption spectra of different cascade reactions run at $T \sim 25^\circ\text{C}$ consisting of various amounts of initially added D-glucose and fixed amounts of GOD and HRPC, recorded after $t = 24$ h. [AOT] = 3.0 mM; [aniline]₀ = 4.0 mM; [D-glucose]₀ = 0 (1), 2.5 (2), 4.5 (3), 25 (4), 100 (5), 250 (6), or 500 mM (7); [GOD] = 0.30 μM ; [HRPC] = 0.92 μM ; pH = 4.3 (100 mM NaH_2PO_4). **a** Measured spectra. **b** A_{1000} , A_{500} , A_{420} , and A_{1000}/A_{500} vs. [D-glucose]₀. The lines between the data points are drawn for guiding the eyes

Reaction optimization upon replacing H_2O_2 by D-glucose and GOD ("cascade reaction")

A few initial trials showed that incubation of D-glucose and GOD with aniline (4.0 mM) and HRPC (0.92 μM) in the presence of AOT vesicles (3.0 mM AOT) at pH = 4.3 (100 mM NaH_2PO_4) at $T = 25^\circ\text{C}$ leads to a change in the color of the reaction mixture from initially colorless to dark blue and finally dark green. This was a first qualitative indication that PANI-ES formation from aniline through the enzymatic cascade reaction with D-glucose, GOD and HRPC is possible. Therefore, in a next step the concentrations of D-glucose and GOD were varied in a systematic way and the UV/Vis/NIR absorption spectra of the different reaction mixtures were recorded after $t = 24$ h for determining

Fig. 2 UV/Vis/NIR absorption spectra of different cascade reactions run at $T \sim 25^\circ\text{C}$ consisting of various amounts of GOD and fixed amounts of initially added D-glucose and HRPC, recorded after $t = 24$ h. $[\text{AOT}] = 3.0$ mM; $[\text{aniline}]_0 = 4.0$ mM; $[\text{D-glucose}]_0 = 100$ mM; $[\text{GOD}] = 0$ (1), 0.01 (2), 0.05 (3), 0.10 (4), 0.15 (5), 0.20 (6), 0.30 (7), 0.40 μM (8); $[\text{HRPC}] = 0.92$ μM ; $\text{pH} = 4.3$ (100 mM NaH_2PO_4). **a** Measured spectra. **b** A_{1000} , A_{500} , A_{420} and A_{1000}/A_{500} vs. $[\text{GOD}]$. **c** Dependence of the concentration of remaining aniline after $t = 24$ h and 14 d on $[\text{GOD}]$. **d** Comparison between the spectrum recorded after $t = 24$ h for the "ideal" cascade reaction mixture with D-glucose and GOD at $[\text{D-glucose}]_0 = 100$ mM and $[\text{GOD}] = 0.20$ μM (solid line, curve 6 in **a**) and the spectrum recorded for the "reference reaction" with initially added H_2O_2 (4.5 mM, dashed line, spectrum of Fig. 1); both with $[\text{AOT}] = 3.0$ mM; $[\text{aniline}]_0 = 4.0$ mM, and $[\text{HRPC}] = 0.92$ μM . In **b** and **c**, the lines between the data points are drawn for guiding the eyes. In **c**, the error bars represent standard deviations from two or three measurements

the "ideal conditions." Data for $[\text{GOD}] = 0.3$ μM and varying initial D-glucose concentration (from 0 to 500 mM) are shown in Fig. 1. Corresponding data for $[\text{GOD}] = 0.15$ and 0.03 μM are shown in Figs. S-10 and S-11, respectively. The reactions were carried out at total volumes of 0.5 mL inside 5-mL polypropylene Eppendorf tubes with closed caps under constant tube rotation for facilitating the exchange of O_2 between the air inside the tubes and the vesicular reaction mixture, see "Experimental". For $[\text{GOD}] \leq 0.30$ μM , the reaction dispersion remained stable without precipitation. For $[\text{GOD}] \geq 0.45$ μM , precipitation was observed within 72 h: green precipitate with a transparent, colorless supernatant if the tube rotation was stopped. Based on the spectra shown in Fig. 1a, Figs. S-10 and S-11, we concluded that a substantial amount of D-glucose is required for obtaining reaction products that have similar UV/Vis/NIR spectra like the ones of the "reference reaction" shown in Fig. S-8. For all three GOD concentrations, 100 mM D-glucose seems to be a good compromise, although with 500 mM D-glucose the A_{1000}/A_{500} value is higher than in the case of 100 mM, 2.3 vs. 1.9 (Fig. 1b).

In a next step, several reaction mixtures were prepared in which the initial concentration of D-glucose was kept constant at $[\text{D-glucose}]_0 = 100$ mM, and the concentration of GOD was varied between 0 and 0.40 μM in order to find the ideal GOD concentration. Again, the UV/Vis/NIR spectrum was recorded after $t = 24$ h and taken as a measure for the formation of PANI-ES products, see Fig. 2. Only for $[\text{GOD}] = 0.20$, 0.30, and 0.40 μM the aniline conversion after $t = 24$ h was comparably high as for the "reference reaction" (90–95%) (Junker et al. 2012). For $[\text{GOD}] < 0.20$ μM , the concentration of remaining aniline levels off after about $t = 60$ min at values which correspond to conversions of $\sim 77\%$ (for $[\text{GOD}] = 0.10$ μM , $\sim 64\%$ (for $[\text{GOD}] = 0.05$ μM), or $\sim 36\%$ (for $[\text{GOD}] = 0.02$ μM), see Figs. 2c and S-12. We therefore considered $[\text{GOD}] = 0.20$ μM as sufficient, ideal concentration. In Table 1, the initial conditions for the ideal reaction mixture

Table 1 Cascade reaction conditions which we considered ideal for the reproducible synthesis of PANI-ES products from aniline and D-glucose with GOD and HRPC in the presence of AOT vesicles as templates at $T \sim 25$ °C for a reaction time of $t = 24$ h (with rotating reaction tubes)

Component	Concentration
AOT	3.0 mM
Aniline	4.0 mM
D-Glucose	100 mM
GOD	0.20 μ M
HRPC	0.92 μ M
$\text{NaH}_2\text{PO}_4 + \text{H}_3\text{PO}_4$	100 mM
H_3O^+	$10^{-4.3}$ M = 50 μ M (pH = 4.3)

For details about the stock solutions and the vesicle dispersion used, and for volumes and order of adding the different stock solutions and the vesicle dispersion, see section “[Experimental](#)”

are summarized. A comparison of the UV/Vis/NIR spectrum of this “ideal reaction mixture,” recorded after $t = 24$ h, with the spectrum of the “reference spectrum,” again recorded after $t = 24$ h, is shown in Fig. 2d. Although both spectra are similar, they are clearly different. For the “reference spectrum” the intensity of the absorption around $\lambda = 1000$ nm is higher and at about $\lambda = 700$ nm lower, if compared to the “ideal” D-glucose/GOD system.

Spectroscopic changes and aniline consumption for the cascade reaction

Changes of the UV/Vis/NIR absorption spectrum of the reaction mixture were measured during the reaction for the “ideal reaction” run in rotating tubes under the elaborated optimal conditions listed in Table 1, see Fig. 3. During the first phase of the reaction, there was a rapid increase in absorbance at $\lambda_{\text{max}} \approx 750$ nm (Fig. 3a, c), correlating with the observed appearance of a blue color at the initial stage of the reaction. The intensity of this absorption band then slowly decreased with reaction time with simultaneous increase in absorption in the NIR region, centered around $\lambda_{\text{max}} \approx 1000$ nm (Fig. 3b, d), correlating with the developing green color of the reaction mixture. Overall, these changes are very similar to the changes observed for the “reference reaction” (Junker et al. 2012). There is, however, also a notable, clear difference between the two systems. The initial increase in A_{750} is much faster for the reference reaction mixture with added H_2O_2 (4.5 mM, no D-glucose, no GOD) (Junker et al. 2012) than for the D-glucose/GOD system. Furthermore, while the spectrum for the reaction mixture with added H_2O_2 remained relatively stable after $t = 24$ h (Junker et al. 2012; Fujisaki et al. 2019), the spectrum of the cascade reaction mixture with D-glucose and GOD still

changed with time after $t = 24$ h, with a decrease in A_{1000} and a considerable broadening of the absorption into the visible as well as the NIR region, see Fig. S-13.

Continuous rotation of the reaction tube during the reaction has a clear positive effect on the reaction (Fig. S-14). After a reaction time of $t = 24$ h, the intensity of the absorption spectrum of the reaction mixture which was not rotated was much lower than in the case of tube rotation. The effect of letting the reaction tube standing, without any rotation, is also obvious from the UV/Vis/NIR spectra recorded during the reaction (Fig. S-15).

The cascade reaction was also run in air-exposed reaction vessels (open tube or flask to maintain a constant O_2 concentration) (Fig. S-16). Obviously, in this case rotation of the reaction vessels in the way done for the closed tubes (Fig. S-2) was not possible. They were left standing at $T \sim 25$ °C for $t = 24$ h. As a result from these measurements, there was no improvement in terms of A_{1000} for the *standing* and *open* reaction mixture if compared to the *rotating* reaction mixture in the *closed* tube (Fig. S-16). For this set of experiments, the pH value in the reaction mixtures was also determined. It dropped in all cases from (unbuffered) pH = 4.3 at the start of the reaction to pH = 3.9 (for the reaction mixture which was kept *standing* in an open vessel), pH = 3.8 for *standing* and *closed*, and pH = 3.6 for *rotating* and *closed* (Table S-1). This difference in measured pH values correlates with the absorption intensity of the reaction mixtures in the Vis/NIR region of the spectrum: lower pH value for reaction mixtures with higher absorbance at $t = 24$ h, i.e., higher reaction yield (as a result of higher amounts of H_2O_2 and D-gluconic acid formed).

The amount of remaining aniline in the cascade reaction mixture was measured for reaction mixtures that were kept inside closed tubes which were rotated and then compared with the same reaction mixtures placed inside closed tubes without rotation. Without rotation, the reaction was slower and leveled off with considerably lower aniline conversion: for $[\text{GOD}] = 0.2$ μ M, about 50% of the initially added aniline remained after $t = 24$ h without tube rotation, as compared to about 10% with tube rotation. (Fig. S-17). This is different to what we observed for the reference reaction with initial addition of H_2O_2 (no D-glucose, no GOD), where reaction tube rotation did not have a significant influence on the outcome of the reaction (see Fig. S-9). It seems that tube rotation facilitates the reaction most likely through a more efficient molecular oxygen exchange between the air in the tube and the reaction mixture. Unless specified, all further measurements were carried out with reaction tube rotation. For the rotating “ideal reaction mixture” (with $[\text{GOD}] = 0.20$ μ M), the time-dependent changes of the aniline consumption in the reaction mixture are shown in Fig. 4. Additional data for $[\text{GOD}] = 0.01, 0.05, 0.10$, and 0.40 μ M are given in Fig. S-12.

Fig. 3 UV/Vis/NIR absorption spectra of samples which were withdrawn at predetermined times from the ideal cascade reaction mixture (see Table 1). The reaction times were $t=0$ s (1), 15 s (2), 30 s (3), 1 min (4), 3 min (5), 10 min (6), 60 min (7), 5 h (8) and 24 h (9). **a**, **b** Measured spectra for $t=0$ –10 min and 10 min–24 h, respectively. **c**, **d** Time-dependent changes of A_{1000} , A_{750} , A_{500} , A_{420} , and A_{300} . In **c** and **d**, the lines between the data points are drawn for guiding the eyes. Please note that the spectra measured for $t=15$ s and $t=30$ s are only approximate spectra due to the rapid changes during the first phase of the reaction, also taking place during the recording of the spectra

Quantification of in situ formed H_2O_2 for the cascade reaction

The concentration of H_2O_2 that (i) formed from D-glucose and O_2 with GOD and (ii) simultaneously was consumed during the HRP-catalyzed oxidation of aniline was determined for aliquots taken from the "ideal cascade reaction mixture" (Table 1) during the course of the reaction (Fig. 5). The quantification was carried out with the spectrophotometric Ti-TPyP assay, using oxo[5,10,15,20-tetra(4-pyridyl)porphyrinato]titanium(IV), as described before (Junker et al. 2012), see "Experimental". Very different from the "reference reaction"—where the concentration of the initially added H_2O_2 was as high as 4.5 mM—the determined concentration of H_2O_2 in the "ideal cascade reaction mixture" was always below 30 μM (Fig. 5). This was also the case for $[\text{GOD}] = 0.10$ or $0.05 \mu\text{M}$, while for $[\text{GOD}] = 0.30$ or $0.40 \mu\text{M}$, the H_2O_2 concentrations were significantly higher, even exceeding 1.0 mM in the case of $[\text{GOD}] = 0.4 \mu\text{M}$ (Fig. S-18).

Changes in GOD and HRP activities and quantification of remaining D-glucose for the cascade reaction

For the "ideal cascade reaction mixture," the activities of HRP and GOD were measured during the course of the reaction by withdrawing small volumes from the reaction mixture and analyzing them with either $\text{ABTS}^{2-}/\text{H}_2\text{O}_2$ (HRPC assay) or D-glucose/ O_2 / ABTS^{2-} /HRP (GOD assay), see Fig. 6 (HRP activity) and Fig. 7 (GOD activity). For HRP, the activity decreased rapidly during the formation of PANI-ES products, very similar to the case of the reference reaction (Fig. 6). The latter data are in good agreement with what was observed before (Junker et al. 2012). The inactivation of HRP is a consequence of the oxidation of aniline (Junker et al. 2012). Without this reaction, HRP is stable (Fig. S-5B). In the case of GOD, the activity also dropped rapidly during the formation of PANI-ES (Fig. 7). After 10 min reaction time, no GOD activity could be detected anymore with the method used. Like in the case of HRP, GOD inactivation is a consequence of

Fig. 4 Time-dependent changes of the concentration of remaining aniline in the "ideal cascade reaction mixture" (see Table 1), as determined by analyzing samples withdrawn from the reaction mixture, see section "Experimental". The lines between the data points are drawn for guiding the eyes. The error bars represent standard deviations from two or three measurements

Fig. 5 Quantification of the net concentration of in situ formed and consumed H_2O_2 for the "ideal cascade reaction mixture" (see Table 1) during the course of the reaction, as determined by analyzing samples withdrawn from the reaction mixture, see section "Experimental". The error bars represent standard deviations from three measurements

the oxidation of aniline, since without aniline, GOD is stable (Fig. S-6B). The rapid loss in enzyme activities in the "ideal cascade reaction mixture" can be compared with the observed rapid consumption of aniline during the initial phase of the reaction (Fig. 4). The enzymatic oxidation of aniline (see Scheme 2) is completed after less than 30 min, while spectroscopic changes due to non-enzymatic processes still take place for several hours (Fig. 3). The rapid loss of

Fig. 6 Changes in the relative activity of HRP during the course of the reaction for the "ideal cascade reaction mixture" (filled symbols, see Table 1) and of the "reference reaction mixture" (empty symbols), as determined by analyzing samples withdrawn from the reaction mixtures. Hundred percent activity refers to the initial rate of reaction at time zero. The error bars represent standard deviations obtained from three measurements, see section "Experimental". The lines between the data points are drawn for guiding the eyes

Fig. 7 Changes in the relative activity of GOD during the course of the reaction for the "ideal cascade reaction mixture" (see Table 1), as determined by analyzing samples withdrawn from the reaction mixture, see section "Experimental". Hundred percent activity refers to the initial rate of reaction at time zero. The error bars represent standard deviations obtained from three measurements. The lines between the data points are drawn for guiding the eyes

GOD activity is in agreement with the low amounts of H_2O_2 detected in the samples withdrawn from the "ideal reaction mixture" (Fig. 5). Without catalytically active GOD, no H_2O_2 can form from D-glucose.

The change of the initially present amount of D-glucose in the "ideal cascade reaction mixture" (100 mM) was

Fig. 8 Quantification of the concentration of D-glucose in the "ideal cascade reaction mixture" (see Table 1), as determined by analyzing samples withdrawn from the reaction mixture, see section "Experimental". Error bars represent standard deviations obtained from three measurements

determined during the reaction by withdrawing small volumes from the reaction mixture, then eliminating GOD and HRP by ultrafiltration, and finally analyzing the ultrafiltrate for D-glucose content, see "Experimental". As seen from the results shown in Fig. 8, after an initial consumption of 5 mM D-glucose, the D-glucose concentration in the cascade reaction mixture remained at about 95 mM. This initial D-glucose consumption and the discontinuity of D-glucose oxidation correlate well with the observed inactivation of GOD (see Fig. 7).

In summary, from previous investigations of the "reference reaction," it is clear that HRP inactivation occurs during the formation of PANI-ES products from aniline (Junker et al. 2012). For GOD, the situation is basically the same. Native GOD is inactivated during PANI-ES product formation.

EPR spectrum of the PANI-ES products obtained from aniline with the cascade reaction

Based on the UV/Vis/NIR absorption measurements, it is expected that the PANI-ES products obtained from aniline with the enzymatic cascade reaction contain unpaired electrons (see Introduction). Therefore, the products should show EPR spectra. This was confirmed by analyzing three different cascade reaction mixtures of the composition shown in Table 1 after $t = 24$ h, with the only exception that the GOD concentration was either 0.09, 0.15, or 0.30 μM instead of 0.20 μM , the "ideal" concentration. There is no doubt that the PANI-ES products of the enzymatic cascade reaction contain paramagnetic centers. The spectrum for $[\text{GOD}] = 0.15 \mu\text{M}$ measured after $t = 24$ h is shown in Fig. 9,

Fig. 9 EPR spectra of (1) a "cascade reaction" run at $T \sim 25^\circ\text{C}$ and recorded after $t = 24$ h. $[\text{AOT}] = 3.0 \text{ mM}$; $[\text{aniline}]_0 = 4.0 \text{ mM}$; $[\text{D-glucose}]_0 = 100 \text{ mM}$; $[\text{GOD}] = 0.15 \mu\text{M}$; $[\text{HRPC}] = 0.92 \mu\text{M}$; $\text{pH} = 4.3$ (100 mM NaH_2PO_4); (2) Control: "EPR spectrum" of the reaction mixture used for the cascade reaction before starting the reaction (no GOD added); (3) the "reference reaction" run at $T \sim 25^\circ\text{C}$ and recorded after $t = 24$ h. $[\text{AOT}] = 3.0 \text{ mM}$; $[\text{aniline}]_0 = 4.0 \text{ mM}$; $[\text{HRPC}] = 0.92 \mu\text{M}$; $[\text{H}_2\text{O}_2]_0 = 4.5 \text{ mM}$; $\text{pH} = 4.3$ (100 mM NaH_2PO_4)

together with the EPR spectrum of the "reference reaction mixture," also measured after $t = 24$ h. Both spectra are very similar, with only slightly different G-factors (2.0063 for the cascade reaction and 2.0064 for the reference reaction, respectively). The EPR signal intensity for the PANI-ES products obtained with the reference reaction was higher than in the case of the PANI-ES products obtained with the cascade reaction (Fig. 9). The EPR signal intensities correlate with the higher absorption in the NIR region for the reference reaction mixture, as compared to the cascade reaction mixture: $A_{1000}(\text{reference}, L = 1 \text{ mm}, t = 24 \text{ h}) = 1.2$ (Fig. 2d) and $A_{1000}(\text{cascade}, L = 1 \text{ mm}, t = 24 \text{ h}) = 0.8$ (see curve 5 in Fig. S-10), respectively.

Comparison of the PANI-ES products obtained with the "ideal cascade reaction" and with the "reference reaction"

The question we asked ourselves at the beginning of the investigation was: can PANI-ES products be obtained in the presence of AOT vesicles as templates at $\text{pH} = 4.3$ in high yield as stable aqueous suspension from aniline with D-glucose, GOD and HRP (and dissolved O_2)? And, if the answer would be "yes": How do the spectroscopic properties of the PANI-ES products obtained with this cascade reaction under "ideal" conditions compare with those resulting from a similar (optimized) reaction without using D-glucose and GOD but addition of H_2O_2 at the beginning of the reaction ("reference reaction")? Although both reactions involve the

use of enzymes as catalysts, the key difference between the two systems is the in situ formation of H_2O_2 from D-glucose with GOD in the case of the cascade reaction, as compared to the manual addition of H_2O_2 at the beginning in the case of the reference reaction.

In this work, we have shown that conditions can be found for the desired GOD- and HRPC-catalyzed synthesis of PANI-ES products with an aniline conversion of ~90% (Fig. 4), similarly to the "reference reaction" (Junker et al. 2012). With the experimental conditions which we considered ideal for the cascade system (Table 1), the products obtained had UV/Vis/NIR absorption and EPR spectra that are typical for PANI-ES products (Figs. 2d, 9). The key spectroscopic differences between the two systems can be summarized as follows:

(i) The absorption of the obtained PANI-ES products at $\lambda \sim 750$ nm, A_{750} , was higher for the cascade reaction than for the reference reaction. In contrast, A_{420} and A_{1000} for the cascade reaction were lower than for the reference reaction (Fig. 2d).

(ii) Although the EPR spectra were very similar, the EPR signal intensity for the PANI-ES products obtained with the cascade reaction was lower than in the case of the reference reaction (Fig. 9).

The observations (i) and (ii) support the previous conclusions that electronic PANI-ES transitions involving unpaired electrons are centered around $\lambda = 420$ and $\lambda \geq 800$ nm, see [Introduction](#).

Conclusions

There is no doubt that PANI-ES products can be obtained in aqueous 0.1 M NaH_2PO_4 solution (pH = 4.3) in the presence of AOT vesicles in a two-enzyme cascade reaction from aniline, D-glucose, GOD and HRPC (and dissolved O_2) at $T \sim 25$ °C within $t = 24$ h. However, there is some drawback of reaction efficiency: GOD is rapidly inactivated during the reaction (Fig. 7). High concentrations of GOD (0.2 μM) and D-glucose (100 mM) are required for achieving high aniline conversion (Table 1). Substantial stabilization of GOD via chemical modification(s) is unlikely. HRPC (0.92 μM) is also rapidly inactivated during the reaction (Fig. 6) (Junker et al. 2012), and attempts to significantly stabilize the enzyme were unsuccessful (Junker et al. 2013). Further studies on the enzymatic synthesis of conducting PANI, for example by using an AOT/SDS (sodium dodecylsulfate) bicontinuous cubic phase as template (Jin et al. 2020), are certainly worth pursuing. Depending on application, enzyme inactivation might not be a crucial problem (Zhang et al. 2020), because enzymes are biological and biodegradable macromolecules. Preliminary experiments showed that the cascade reaction we elaborated is a useful extension to the reference reaction applied so far in the study of artificial

cell-like systems, as briefly mentioned in the introduction (Kurusu et al. 2019).

Our investigations can be compared to previous reports on the synthesis of PANI from aniline with D-glucose/GOD (no HRP, no template) (Kausaite et al. 2009; German et al. 2017) or from aniline with D-glucose and a mixture of GOD and hemoglobin immobilized on a glassy carbon electrode consisting of adsorbed carboxylic acid functionalized polystyrene (Zhang et al. 2013). A direct comparison is, however, difficult. The aim of this previous work and the reaction conditions were very different from ours. Moreover, the products obtained did not have the characteristic spectra of PANI-ES, and the PANI products obtained precipitated as micrometer-sized particles (Kausaite et al. 2009; German et al. 2017). This is very different from the work we describe here, where prevention of product precipitation was set as one of the goals of the work, successfully achieved by the use of AOT vesicles as soft, dispersed additives that promote and guide the enzymatic cascade reaction (Serrano-Luginbühl et al. 2018).

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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