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Enzymatic Synthesis of Highly Electroactive Oligoanilines from a *p*-Aminodiphenylamine / Aniline Mixture with Anionic Vesicles as Templates

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ABSTRACT

Oligoanilines with characteristic properties of the electrically conductive emeraldine salt form of polyaniline (PANI-ES) are promising molecules for various applications. A mixture of such oligoanilines can be obtained, for example, enzymatically under mild conditions from the linear aniline dimer *p*-aminodiphenylamine (PADPA) with hydrogen peroxide (H_2O_2) and low amounts of horseradish peroxidase (HRP) in an aqueous pH = 4.3 suspension of anionic vesicles formed from AOT, the sodium salt of bis(2-ethylhexyl)sulfosuccinate. However, the simultaneous formation of undesired side products containing phenazine-type units or oxygen atoms is unsatisfactory. We have found that this situation can be improved considerably by using a mixture of PADPA and aniline instead of PADPA only, but otherwise nearly identical conditions. The PANI-ES-like oligoaniline products which are obtained from the PADPA & aniline mixture were not only found to have much lower contents of phenazine-type units and do not contain oxygen atoms, but also were shown to be more electroactive in cyclic voltammetry measurements than the PANI-ES-like products obtained from PADPA only. The AOT vesicle suspension remained stable without product precipitation during and after the entire reaction so that it could be analyzed by *in situ* UV/vis/NIR, *in situ* EPR, and *in situ* Raman spectroscopy measurements. These measurements were complemented with *ex situ* HPLC analyses of the deprotonated and reduced products formed from mixtures of PADPA and either fully or partially deuterated aniline. Based on the results obtained, a reaction mechanism is proposed for

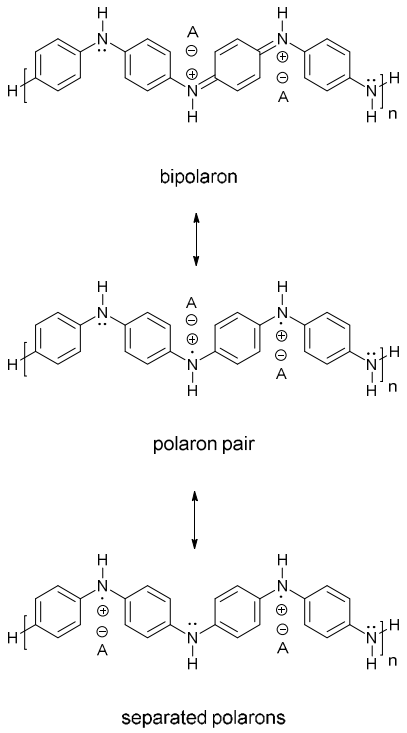
explaining this improved HRP-triggered, vesicle assisted synthesis of electroactive PANI-ES-like products. The oligomeric products obtained can be further used, without additional special workup, for example to coat electrodes for their possible application in biosensor devices.

1. INTRODUCTION

Enzymatic polymerizations have been investigated for many years to explore whether synthetic polymers with desired and defined chemical structures and useful physico-chemical properties can be obtained from a chosen monomer with an appropriate enzyme as catalyst.[1-8] The use of enzymes for the synthesis of polymers is an alternative approach to purely chemical (or electrochemical) methods and may have several advantages because enzymatic polymerization reactions often occur regio- and stereoselectively, and can be carried out under mild and environmentally friendly conditions. There are, however, also severe limitations in using enzymes. The stability and the costs of the enzyme are critical issues, as well as restrictions with respect to the chemical structure of the monomers that can be polymerized enzymatically. Furthermore, in many cases, neither the chemical structures of the obtained products are known in detail, nor have the reaction mechanisms and the actual role of the enzyme in the reaction been clarified. Therefore, fundamental studies on enzymatic polymerizations which deal with these basic issues are as important as application-oriented investigations.

One of the most intensively investigated enzymatic polymerizations is the one of aniline[9-15] to yield polyaniline (PANI) in its conductive, half-reduced and protonated half-oxidized emeraldine salt form (PANI-ES), with delocalized unpaired electrons on every second nitrogen atom and fully aromatic benzene rings, the so-called polaron state.[16-24] The chemical structure of a constitutional repeating unit of PANI-ES with bipolarons, separated polarons or polaron pairs is

shown in **Scheme 1**. This is a highly idealized representation of the actual PANI-ES chains obtained. Not all chains of PANI-ES will have the same length, and it is expected that not all chains will be composed of linear N−C-*para* coupled units only. Similarly to chemically or electrochemically synthesized PANI-ES,[22,23] structural defects may be present as well, *e.g.* phenazines or branched products which originate from an *ortho* coupling of the aniline units.



Scheme 1. Chemical structure representations of an ideal PANI-ES molecule with N−C-*para* coupled aniline units with either bipolarons, polaron pairs, or separated polarons;[21,24] A^- is the counter ion, n represents the number of repeating units. The oligomer with n = 1 is the linear N−C-*para* coupled aniline tetramer in its emeraldine salt form. It is the same molecule as the linear N−C-*para* coupled dimer of PADPA in its emeraldine salt form, $(PADPA)_2$-ES.

Polymers or oligomers with characteristic features of polaronic PANI-ES units - with a maximal absorption, λ_{max}, at ≈1000 nm (assigned to the $\pi \rightarrow$ polaron transition) and ≈ 420 nm (polaron $\rightarrow \pi^*$ transition)[25,26] - can be obtained from aniline with oxidative enzymes and enzyme-specific oxidants, *e.g.,* a peroxidase and H_2O_2 or a laccase and O_2.[9-15] Conceptually, there are two conditions which need to be fulfilled for a successful enzymatic polymerization of aniline into PANI-ES products. One is related to the property of the enzyme, the other to the property of the reaction medium. First, in a mediator-free system,[7] the monomer (aniline) must be able to reach the active site of the enzyme. Furthermore, the oxidative power of the enzyme/oxidant system under the experimental conditions must be high enough so that aniline can be oxidized by the enzyme/oxidant system. Second, the experimental conditions must be screened by varying the reaction parameters (*e.g.* the pH and the monomer, enzyme, or oxidant concentrations) to elaborate whether conditions exist at which products with the desired PANI-ES properties can be obtained. With respect to the second prerequisite, many previous studies have shown that for obtaining PANI-ES products with an oxidative enzyme, the presence of a "chemical structure-controlling template"[13] is essential. These "templates" are anionic additives containing covalently bound anionic functional groups, which are dispersed in an aggregated state in the reaction medium: anionic polymers,[9-15] anionic micelles[9-15] or anionic vesicles.[13,15] Among the most effective "templates" are sulfonated polystyrene (SPS),[27,28] micelles of sodium dodecylbenzenesulfonate (SDBS),[29,30] or vesicles of sodium bis(2-ethylhexyl)sulfosuccinate (known as AOT).[31,32] They all represent soft sulfonate-rich interface systems.

Previously, our work was focused on basic investigations of the effect of AOT vesicles on the polymerization of aniline with either horseradish peroxidase isoenzyme C (HRP) and H_2O_2[31] or

with *Trametes versicolor* laccase (TvL) and O_2.[32] More recently, instead of aniline, the N−C-*para* coupled aniline dimer *p*-aminodiphenylamine (PADPA) was used, again with AOT vesicles and either HRP/H_2O_2[33] or TvL/O_2.[34-36] The use of PADPA has two important advantages. First, the as-obtained products are not true polymers but oligomers that can be deprotonated, then extracted into an organic solvent and chemically reduced, such that a separation and analysis of them by HPLC-MS is possible.[33,36] Second, the reaction with PADPA can be kept runniung with much less enzyme than in the case of aniline.[33,34] This is most likely directly related to the fact that the products obtained from PADPA are only oligomers, *i.e.,* they are much shorter than the products obtained from the polymerization of aniline. Nevertheless, in both cases – with HRP/H_2O_2 or with TvL/O_2 – the products obtained under optimal conditions contain a high amount of polaronic PANI-ES-like units. With TvL/O_2 and PADPA, the main product from the reaction at optimal conditions is the linear tetraaniline as PANI-ES, *i.e.,* $(PADPA)_2$-ES (**Scheme 1**).[36] The amount of phenazine units formed is low (**Scheme S-1** Supporting Information).[35,36] With HRP/H_2O_2 and the optimal conditions for this reaction, $(PADPA)_2$-ES is also obtained.[33] In this case, however, in this case, the formation of considerable amounts of phenazine units is obvious, and we were unable to prevent this.[33] Furthermore, significant amounts of oxygen-containing products, originating from hydrolytic reactions, were also found.[33]

In the work presented, we investigated whether the quality of the oligomeric reaction products obtained from PADPA and HRP/H_2O_2 with AOT vesicles as templates can be improved through the use of a *mixture of the two monomers*, PADPA & aniline. We particularly wondered whether the use of PADPA and aniline together would not only lead to a lower content of phenazine units, but also to longer chains with a PANI-ES character and to an increased polaron content, as compared to using PADPA only. This turned out to be the case to some extent. More

importantly, however, we found that the amount of phenazine units in the products decreased and that the products showed a higher redox activity than the ones obtained with HRP/H_2O_2 from PADPA alone under otherwise similar conditions. Moreover, oxygen-containing products did not form by using the PADPA & aniline mixture.

2. MATERIALS AND METHODS

2.1. Chemicals. Horseradish peroxidase isoenzyme C (HRP, 278 Umg^{-1}, Lot. number 2131616000) was purchased from Toyobo Enzymes. The HRP concentration was determined spectrophotometrically by using $\varepsilon_{403} = 1.02\times10^5$ M^{-1} cm^{-1} as molar absorption coefficient.[37] Sodium bis(2-ethylhexyl)sulfosuccinate (AOT ≥ 99%) and PADPA (*p*-aminodiphenylamine, *N*-phenyl-1,4-phenylene-diamine, 98%, recrystallized from hexane)[34] were purchased from Sigma-Aldrich. Aniline (≥ 99.8%) and hydrogen peroxide (H_2O_2, 35 wt% = 11.6 M) were purchased from Acros. D5-Aniline (2,3,4,5,6-pentadeuteroaniline) (98 atom % D) was purchased from Cambridge Isotopes. D1-aniline ((4-^{2}H)-aniline) was synthesized in the same way as described previously.[31,36] All other chemicals used were the same as in our previous work and used as received.[31,33]

2.2. AOT Vesicles. AOT vesicles (20 mM AOT) were prepared with the thin-film hydration freeze-thawing polycarbonate membrane extrusion method by using a pH = 4.3 sodium dihydrogenphosphate solution (0.1 M NaH_2PO_4, pH adjusted with 1 M HCl), as described before.[31] The average hydrodynamic vesicle diameter immediately after preparation was about 80-100 nm, as determined by dynamic light scattering measurements.[31] The vesicle suspension was stored protected from light at room temperature and used within 30 days after preparation.

During this period of time, the vesicle suspension remained colloidally stable without any precipitation.

2.3. Enzymatic Copolymerization of PADPA & Aniline Mixture. Based on the optimal reaction conditions elaborated previously for the oxidation of PADPA with the same type of enzyme-vesicle system (2.0 mM AOT, 1.0 mM PADPA, 30 nM HRP, 1.0 mM H_2O_2),[33] different mixtures of PADPA and aniline were used instead of PADPA alone. The total aniline unit concentration was kept constant at 2.0 mM in order to be comparable with the reaction containing only PADPA at 1.0 mM, and [PADPA] + [aniline] = [H_2O_2], see **Scheme S-2**. As shown in **Figure S-1** (Supporting Information), 30% PADPA replaced by aniline, i.e., 0.7 mM PADPA & 0.6 mM aniline, was found to be optimal for obtaining high intensity in the near infrared region of the absorption spectrum (characteristic for the polaron form of PANI-ES).[25,26] All further measurements were carried out with a mixture of [PADPA] = 0.7 mM and [aniline] = 0.6 mM. This copolymerization of PADPA & aniline with HRP/H_2O_2 as catalyst and oxidant was carried out at pH = 4.3 at room temperature in closed 50 mL glass bottles with a reaction volume of 10 mL. All components of the reaction system, except H_2O_2, were mixed first in the following sequence (optimal conditions, i.e., 0.7 mM PADPA, 0.6 mM aniline): 3.67 mL NaH_2PO_4 solution (0.1 M, pH = 4.3), 1 mL AOT vesicle suspension (20 mM, pH = 4.3), 4.67 mL PADPA stock solution (1.5 mM, prepared in 0.1 M NaH_2PO_4 solution with the pH value being adjusted to 4.3 with 1 M HCl), 150 μL aniline stock solution (40 mM in 0.1 M NaH_2PO_4 solution, pH adjusted to 4.3 with 1 M HCl), 448 μL of a freshly prepared 0.67 μM HRP stock solution. This HRP stock solution was prepared as follows: 3.89 mg HRP powder were first dissolved in 1 mL 0.1 M NaH_2PO_4 solution (pH = 4.3), yielding a HRP concentration of ca. 70 μM (spectrophotometrically determined, stable for three weeks at 4 °C); this concentrated HRP

solution was further diluted with the 0.1 M NaH_2PO_4 solution (pH = 4.3) to obtain the actually used 0.67 μM HRP stock solution. After preparing and gently swirling the vesicle suspension containing PADPA, aniline and HRP, the reaction was triggered by adding 65 μL of a H_2O_2 stock solution (0.2 M, freshly prepared by adding 87.43 μL of 35 wt % H_2O_2 to 5 mL H_2O). The initial concentrations in the reaction mixture were: 2.0 mM AOT, 0.7 mM PADPA, 0.6 mM aniline, 30 nM HRP and 1.3 mM H_2O_2, pH = 4.3 (0.1 M NaH_2PO_4). The typical reaction time was 24-72 h at room temperature (T ≈ 25 °C).

2.4. *In situ* UV/vis/NIR, EPR and Raman Spectroscopy Measurements. Absorption measurements in the ultraviolet (UV), visible (vis) and near infrared (NIR) region of the spectrum were recorded with a JASCO V-670 spectrophotometer at room temperature using quartz cuvettes from Hellma Analytics with optical path lengths of 0.1 cm. To determine the HRP concentration in small volumes (ca. 5 μL) a NanoDrop ND1000 spectrometer from Thermo Scientfic was used. Electron paramagnetic resonance (EPR) measurements of the reaction suspensions were recorded with a flat quartz cell in the same way as described before.[35] Details about the *in situ* Raman spectroscopy measurements can be found in the same publication.[35]

2.5. HPLC-DAD and HPLC-MS Measurements. The oligoaniline products of the oxidation of the reaction mixture with HRP/H_2O_2 were analyzed by HPLC with a diode array detector (DAD) or by a mass spectroscopy (MS) analysis of the eluting peaks (Luginbühl et al., 2016).[36] After a given reaction time, 500 μl of the reaction mixture (initially 10 mL) were removed from the 50 mL glass bottle and added to 1 ml methyl-*t*-butylether (MTBE) and 100 μl of a 25 % (wt/wt) NH_3 solution in an Eppendorf tube. The resulting emulsion was shaken vigorously and left to stand overnight to ensure complete extraction of the products from the aqueous vesicle suspension into MTBE. A volume of 1 mL of the supernatant MTBE solution was removed and

added to 1 ml acetonitrile, followed by removal of MTBE at room temperature and 70 mbar. The products were reduced by adding 400 µL hydrazine (H_4N_2) and stirring vigorously for 10 min. Finally, 1 mL acetonitrile and 8 ml of a 10 mM NH_4HCO_3 buffer solution (pH = 10, adjusted with the NH_3 solution) were added. This acetonitrile-buffer solution was then applied onto the HPLC column (reverse-phase EC 150/4.6 Nucleodur C18 Isis from Machery-Nagel, Switzerland).[36]

2.6. Electrode Coating and Cyclic Voltammetry Measurements. For cyclic voltammetry (CV) measurements, a commercial glassy carbon (GC) electrode (from Metrohm, Herisau, Switzerland) with a diameter of 3 mm, encased in Teflon, was used. The electrode was coated with the enzymatically prepared PANI-ES-type vesicle suspension obtained under optimal conditions from PADPA and aniline (see 2.3.) by following a procedure which is based on a previously reported one.[33,35] The electrode was first modified by removing about 1 mm of the GC with a small diamond drill in order to create a shallow well within the exposed area of the GC disk. In this way, the deposited reaction suspension is not spilled over the Teflon jacket but only covers the GC, leading to a more reproducible coating. Before and after coating the electrode with the reaction suspension, the electrode was cleaned by polishing with 1 µm diamond paste, rinsing with deionized water and drying with methanol. Sometimes, if this procedure was not sufficient, left-over coating was removed with 90 % sulfuric acid. Then, 4 µL of the reaction suspension was drop-cast onto the GC electrode, which was then turned upside down and suspended over a simple tungsten lamp to dry for ca. 3 min. This was done twice (2 x 4 µL = 8 µL of the reaction suspension per measurement). CV measurements were carried out using a GC counter-electrode and a Ag/AgCl reference electrode, in a 0.1 M NaH_2PO_4 solution (pH = 4.3). Voltage sweeps were conducted from +600 mV to – 600 mV, at a speed of 100

mV/s; three sweeps per sample which were then later averaged. The cyclic voltammogram generated by the uncoated electrode immediately before coating was used as a background signal and subtracted from the signal generated by the coated electrode.

3. RESULTS AND DISCUSSION

3.1. Optimal Reaction Conditions for Obtaining PANI-ES-type Products from a PADPA & Aniline Mixture With HRP/H_2O_2 and AOT Vesicles. A systematic investigation of the type of reaction mixture we have explored in the work presented is a big challenge since the outcome of the reaction depends on many factors. For finding reaction conditions which we consider "optimal", we started with two reference reactions. Both contained AOT vesicles as "templates" at pH = 4.3 (aqueous 0.1 M NaH_2PO_4) with HRP/H_2O_2 as enzyme/oxidant system. The first reference reaction was the one described previously for the PADPA monomer.[33] In the second reference reaction, aniline was the monomer used.[31] There is a key requirement for the mixture of PADPA & aniline. It is based on characteristic features of the absorption spectrum of the conductive polaron form of PANI-ES. The polaron form of PANI-ES (**Scheme 1**) has absorption bands in the NIR region of the electromagnetic spectrum, at about 1000 nm (assigned to the $\pi \rightarrow$ polaron transition) and at about 420 nm (polaron $\rightarrow \pi^*$).[25,26] So, the reaction conditions were varied to see whether reaction products with these characteristic spectroscopic features can be obtained from a mixture of PADPA and aniline with HRP/H_2O_2 in the presence of AOT vesicles. We set these further requirements: (i) low absorption at about 600 nm (originating from unwanted phenazine side products),[33] (ii) high monomer conversion, (iii) use of low amounts of HRP (for economic reasons, **Fig. S-2**), and (iv) no product precipitation (stable suspension for

possible direct applications without any subsequent purification steps, *e.g.* for the coating of electrodes).[35] Based on our previous investigations with HRP/H_2O_2 and aniline[31] or PADPA,[33] a pH = 4.3 solution was used for all reaction conditions tested (aqueous 0.1 M NaH_2PO_4) at room temperature (T ≈25 °C). During the optimization procedure, various fractions of PADPA were replaced by aniline, whereby the total aniline unit concentration was kept constant at 2.0 mM, to ensure that the reactions of these mixtures were comparable with the reaction of PADPA alone, where [PADPA] = 1.0 mM, equivalent to an aniline unit concentration of 2.0 mM (see **Fig. S-1**).[33] The best conditions we found are 2.0 mM AOT (vesicle suspension), 0.7 mM PADPA, 0.6 mM aniline, 1.3 mM H_2O_2, and 30 nM HRP, see **Figs. S-1** and **S-2**. We refer to these conditions as "optimal reaction conditions".

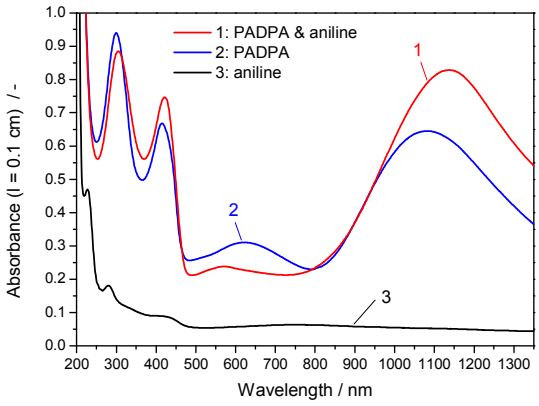
3.2. *In Situ* UV/vis/NIR and EPR Spectroscopy Measurements. Under "optimal reaction conditions", the UV/vis/NIR absorption spectrum was recorded 3 days after starting the reaction at T ≈ 25 °C (curve 1 in **Figure 1a**). For comparison, the spectra obtained with the same HRP concentration from either PADPA alone (curve 2) or from aniline alone (curve 3) are also shown. The spectrum of the products obtained from PADPA alone is in accordance with the spectrum measured previously for the same conditions.[33] Changes in the spectra occurring during the reaction with PADPA alone (**Fig. S-3**) are also similar to previous observations.[33] The spectrum of the products obtained from the mixture of PADPA & aniline (**Fig. 1a**, curve 1) clearly indicates better "product quality" compared to the products obtained from PADPA alone (curve 2): more intense absorption at ≈1000 nm and ≈420 nm, and lower absorption at ≈600 nm. With aniline only, there is no evidence for the formation of desired products; the 30 nM HRP concentration is too small to cause efficient reaction, in agreement with our previous findings.[31] The higher absorption intensities at ≈1000 nm and ≈420 nm – indicative of delocalized polaron

formation[25,26] – correlate with the significantly stronger EPR signal observed for the "optimal reaction conditions" (PADPA & aniline), if compared to the EPR signal of the products obtained from PADPA only (**Fig. 1b**).

The changes of the UV/vis/NIR and EPR spectra recorded for the PADPA & aniline mixture during the reaction are complex and imply that long-lived intermediates are formed (**Fig. 2**). During the first phase of the reaction, a strong absorption band centered on 700 nm appeared (**Fig. 2a**). Its highest intensity was reached after 8-15 min; afterwards, the band steadily decreased with time (**Fig. 2a,b**). The bands typical for PANI-ES, at ≈1000 nm and ≈420 nm, monotonously and slowly increased in intensity with time, as illustrated in **Fig. 2b.** Interestingly, the characteristic NIR band showed up with a maximum at 990 nm before it continuously shifted to a final value of 1140 nm, see **Fig. 2a,b**. A strong band with a maximum at about 600 nm, typical for the formation of phenazine-type structures,[33] did not appear. However, a careful analysis of the absorption intensity between 500 and 600 nm suggested that a weak absorption band still developed in this region at later stages of the reaction, after the band at ≈700 nm had almost completely disappeared, see **Fig. 1a**, curve 1.

The intensities of the EPR spectra which were recorded during the reaction correlate well with the changes observed in the NIR spectra, as expected for the presence of unpaired electrons in the polaron form of PANI-ES-like products.[25,26] The shift in the EPR spectrum at the later stage of the reaction – **Fig. 2c**: EPR spectra recorded after 4 h and after 24 h – correlates with the observed shift in the NIR band maximum (**Fig. 2a,b**).

a)



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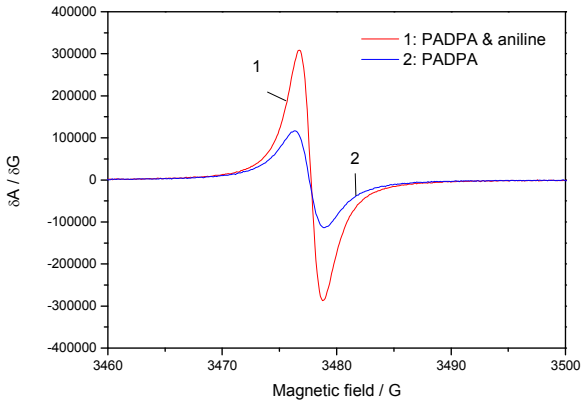
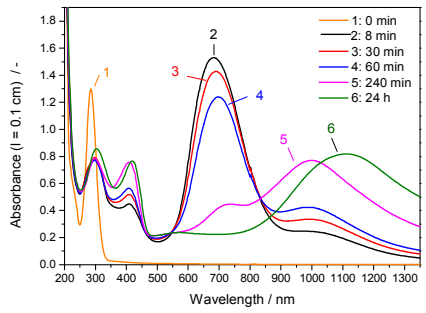


Figure 1. (a) *In situ* UV/vis/NIR and (b) *in situ* EPR spectra of the reaction suspensions with (1) HRP/H_2O_2 in the presence of AOT vesicles and the mixture of PADPA & aniline as monomers (red). [HRP] = 30 nM, [AOT] = 2.0 mM; [PADPA] = 0.7 mM; [aniline] = 0.6 mM; [H_2O_2] = 1.3 mM. (2) Spectrum obtained with the same HRP and AOT concentrations from PADPA only (1.0 mM) and 1.0 mM H_2O_2 (blue). (3) Spectrum from aniline only (2.0 mM) and 2.0 mM H_2O_2 (black); pH =4.3 (aqueous 0.1 M NaH_2PO_4), T ≈ 25 °C. The reaction time for the spectra shown in (a) was 3 days and for those in (b) 1 day.

a)



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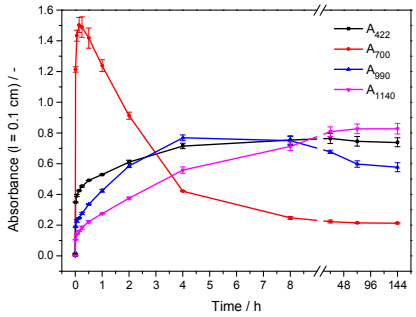


c)

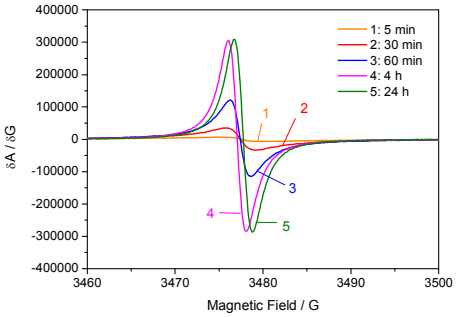


Figure 2. Kinetics of the reaction of the PADPA & aniline mixture with HRP/H_2O_2 in presence of AOT vesicles. The reaction was followed by *in situ* UV/vis/NIR (a, b) and *in situ* EPR spectroscopy (c). (a) The changes in the UV/vis/NIR spectrum are illustrated for reaction times of 0 min (1, orange), 8 min (2, black), 30 min (3, red), 1 h (4, blue), 4 h (5, magenta), and 1 d (6, green); (b) changes of A_{422}, A_{700}, A_{990}, and A_{1140} with reaction time; means and standard deviations from 3 separate reactions using the same stock solutions; (c) EPR spectra recorded after 5 min (1, orange), 30 min (2, red), 1 h (3, blue) 4 h (4, magenta), 1 d (5, green). [AOT] = 2.0 mM, [PADPA] = 0.7 mM, [aniline] = 0.6 mM, [HRP] = 30 nM, [H_2O_2] = 1.3 mM, pH = 4.3 (aqueous 0.1 M NaH_2PO_4), T ≈25 °C.

3.3. *In Situ* Raman Spectroscopy Measurements and Their Correlation with the UV/vis/NIR Measurements. The key results obtained from the Raman spectroscopy analysis are in good agreement with the results of the UV/vis/NIR and EPR measurements.

(i) A broad, strong $\nu(C\sim N^{\bullet+})$ polaron band at 1344 cm^{-1}, which is typical for the conductive form of PANI-ES,[38-41] is present in the spectra of the PADPA & aniline mixture measured after 1 or 3 days, see **Figure 3**. This band developed at later stages of the reaction, in parallel to the increase in absorbance at ≈1000 and ≈420 nm (**Fig. 2a,b**) and to the increase in the EPR signal intensity (**Fig. 2c**). Compared to Raman spectra of the products obtained with HRP/H_2O_2 and AOT vesicles from PADPA alone,[33] the relative intensity of the polaron band at 1344 cm^{-1} for a reaction time of 3 days is higher for the PADPA & aniline mixture (see **Fig. S-4** and the data in **Table S-1**). This is again in good agreement with the EPR results (**Fig. 1b**).

(ii) A band at ≈1440 cm^{-1}, which is attributable to ring C=C stretching vibrations in substituted phenazine- and *N*-phenylphenazine-type oligomers and/or in short branched oligomeric units[33] (*i.e.,* related to 'structural defects'), is seen in the spectra of the PADPA & aniline mixture recorded at later stages of the reaction, but is absent in early stage spectra (**Fig. 3**). This agrees with the development of the UV/vis/NIR spectrum (band at ≈600 nm, **Fig. 1a**). The relative intensity of the Raman band at ≈1440 cm^{-1} is significantly higher for products obtained from PADPA alone (**Fig. 3**, top) compared to the products obtained from the PADPA & aniline mixture (**Fig. 3**, bottom), see also **Table S-1**. This feature, together with the strong, broad band at ≈1344 cm^{-1}, indicates that in products from the PADPA & aniline mixture the relative content of phenazine and branched units in the final products is reduced, resulting in a higher electrical conductivity and a higher electroactivity, as compared to the products obtained from PADPA alone.

Looking at the concurrent changes in the Raman spectrum and in the UV/vis/NIR spectrum (see 3.2.) observed during the course of the reaction, we assume that these changes reflect the same molecular transformations. About their very nature we can deduce some general information from our data, but no details. We particularly tried to correlate the drastic changes in the UV/vis/NIR spectrum of the PADPA & aniline reaction mixture between t = 4 h and t = 24 h with the changes in the corresponding Raman spectra (**Fig. 3**).

The origin of the absorption band at ≈700 nm can be explained by comparison with known materials. Typical $\pi \rightarrow$ polaron transitions of *localized polarons* in *random (twisted) coil conformations* usually are observed at ≈780–850 nm for PANI-ES.[25] The blue shift to ≈700 nm observed in our case may be caused by shorter conjugated chains and/or more twisted conformations.[42,43] It has been reported that the doped phenyl end-capped aniline dimer ($B3^{\bullet+}$, produced by oxidation/doping of *N,N'*-diphenyl-1,4-phenylenediamine, B3) exhibits an absorption band at ≈700 nm, which is, together with a band at 387 nm, typical for the paramagnetic radical cation (polaron) form $B3^{\bullet+}$ of this type of oligomer.[43,44] Thus, the band at 700 nm is most likely a signature of short aniline oligomers with isolated radical cations. The fading of the band at ≈700 nm together with the shift of the $\pi \rightarrow$ polaron band at 990nm (t = 4 h) to 1140 nm (t = 24 h) (**Fig. 2a**) indicates extensive formation of *delocalized* polarons *in longer conjugated chains*, and a *change of chain conformation from twisted coil to extended coil* (more ordered with improved coplanarity of rings).[25]

The most important changes in the Raman spectra at t = 24 h compared to those at t ≤ 4 h (**Fig. 3**) are the following:

(i) There is an abrupt increase of the intensity ratio of the band due to quinonoid (Q) and semiquinonoid (polaronic, SQ) units at ≈1595 cm^{-1} [$\nu(C{=}C)_Q/(\nu(C{\sim}C)_{SQ}$] and the band due to

benzenoid (B) units at ≈1628 cm^{-1} [$\nu(C\sim C)_B$]. For $t \leq 4$ h, the band at 1628 cm^{-1} is stronger than the band at 1595 cm^{-1}, while at t = 24 h the situation is reverse;

(ii) There is a significant growth of the polaron band at 1344 cm^{-1}, indicating extensive formation of delocalized polarons,[38] while the band at ≈1380 cm^{-1} disappears; the latter originates from localized polarons in PANI[38] and is also known for the mentioned doped end-capped aniline dimer $B3^{\bullet+}$.[44] Therefore, we correlate the early phase absorption band at about 700 nm in the UV/vis/NIR spectra (**Fig. 2a**) with the absorption band at about 1380 cm^{-1} in the Raman spectra (**Fig. 3**); both bands belong to localized polarons (radical cations) and short conjugated chains.

(iii) The band at 428 cm^{-1}, attributable to C–N–C torsion (out-of-plane of the B ring motions),[36,45] is very strong and sharp from 30 s to 2 h and has significantly weakened and broadened at t = 24 h, indicating changes in chain geometry/conformation.

(iv) The band at 602 cm^{-1}, attributed to the B/SQ ring deformation (in-plane) in the polaronic PANI-ES structure,[43] appears for the first time at t = 4 h and becomes distinct at t = 24 h, indicating extensive formation of delocalized polarons and more stretched chains.[45] An enhancement of the Raman band at 600 cm^{-1} has been reported for PANI upon 'secondary doping' with camphorsulfonic acid in m-cresol, and this feature has been attributed to changes in chain conformation from random coil to extended coil. As extended coil chains exhibit increased ring coplanarity, facilitating polaron delocalization and providing symmetry of translation, the corresponding vibrational band becomes more intense.[45]

All described changes occurring during the reaction in the *in situ* Raman, UV/vis/NIR and EPR spectra definitely do correlate.

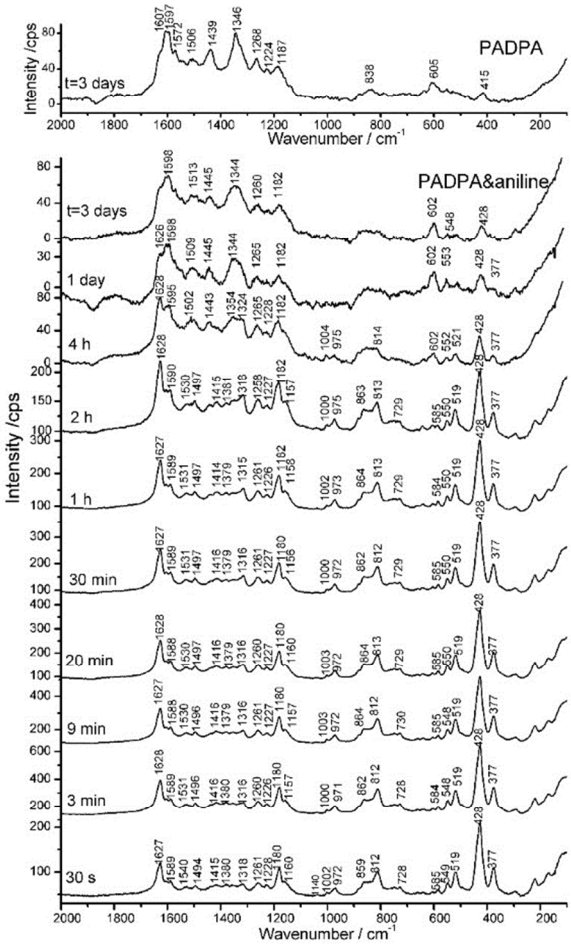


Figure 3. Bottom: Kinetics of the reaction of the PADPA & aniline mixture with HRP/H_2O_2 in the presence of AOT vesicles under optimal reactions conditions monitored with *in situ* Raman spectroscopy at different reaction times. [AOT] = 2.0 mM, [PADPA] = 0.7 mM, [aniline] = 0.6 mM, [HRP] = 30 nM, [H_2O_2] = 1.3 mM, pH = 4.3 (aqueous 0.1 M NaH_2PO_4), T ≈25 °C. A fluorescence background correction (polynomial order: 5) was performed for the spectra recorded at reaction times 4 h, 1 day, and 3 days. Top: For comparison, the *in situ* Raman spectrum of the products obtained after a reaction time of 3 days with HRP/H_2O_2 and AOT vesicles from PADPA alone is also shown (2.0 mM AOT, 1.0 mM PADPA, 30 nM HRP, 1.0 mM H_2O_2, pH = 4.3 (aqueous 0.1 M NaH_2PO_4), 25 °C), taken from Luginbühl et al (2016).[33]

3.4. Reaction Progress and Product Analysis by *Ex Situ* HPLC Measurements. After a given reaction time, 500 µl of the reaction suspensions were treated as described in section 2.5. Information about the oxidation state of *in situ* formed intermediates and final products is lost thereby. The analysis of the extract yields masses and spectra of deprotonated and reduced compounds. This was effected by reverse-phase HPLC, with elution peak detection by either using a diode array detector (DAD) or a mass spectrometer (MS).[33,36] It was possible to monitor the kinetics of the reaction (DAD spectra), and molar masses of the main intermediates and final products were determined by MS. We attempted elucidation of the chemical structures of key intermediates and final products by using D1-aniline and D5-aniline. They were introduced as reactants together with non-deuterated PADPA.

Chromatograms of products after reaction times of 0 min to 3 days, recorded at 310 nm (DAD) are shown in **Figure 4a,** with those chromatographic peaks labeled that we could identify. Aniline eluted at a retention time (rt) of ca. 8 min and PADPA at rt ≈ 21 min. Both monomers are consumed quickly (**Fig. S-5**).

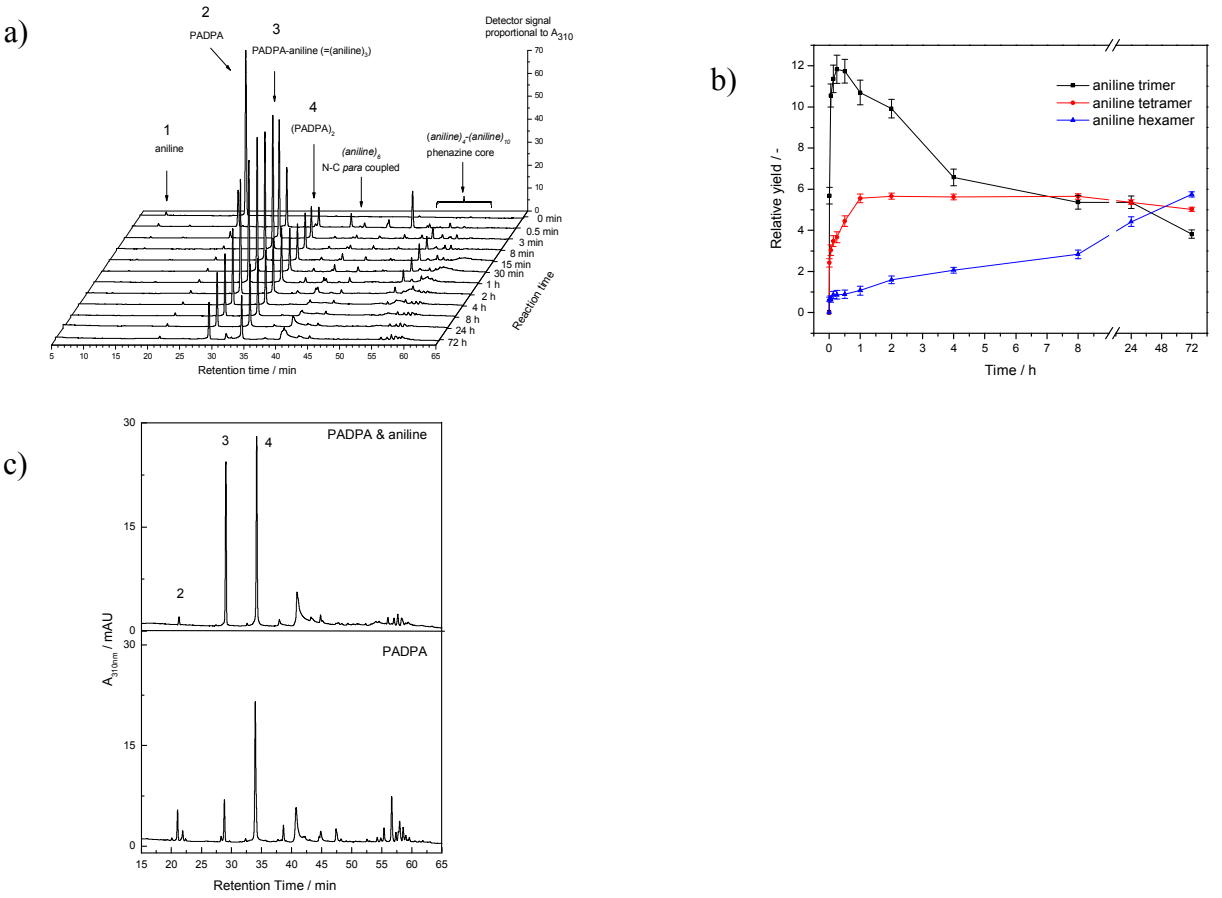
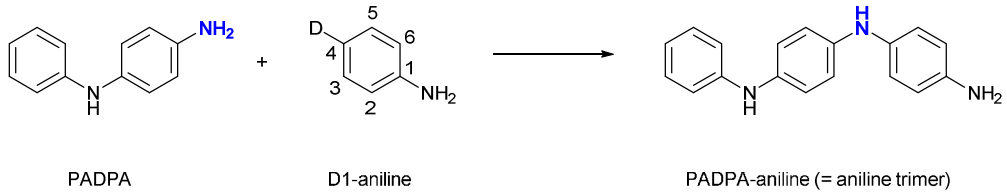


Figure 4. Kinetics of the reaction of the PADPA & aniline mixture with HRP/H_2O_2 in presence of AOT vesicles monitored with HPLC. At selected times, the products were deprotonated, extracted and reduced. (a) Full chromatograms (A_{310} vs. retention time) for different reaction times with peak identifications. Assignments in *italics* mean that structures of these products are incompletely identified. (b) Changes in relative yields of aniline trimer, tetramer, and hexamer. (c) Comparison of the chromatograms after 24 h of the reaction with PADPA & aniline (top); and of the reaction with PADPA only (bottom), see Luginbühl et al. (2017).[33] 2: PADPA; 3: PADPA-aniline (= aniline trimer); 4: $(PADPA)_2$ (= aniline tetramer). Note that formation of the aniline trimer from PADPA only must involve degradation reactions.[33]

At rt ≈ 30 min, a peak appears and increases rapidly during 8 min. From 8 min to 2 h, the peak height remains stable, and afterwards it decreases (**Fig. 4a** and **Fig. 4b**). MS detection revealed that the peak at rt ≈ 30 min belongs to an aniline trimer (**Fig. S-6a**). Further HPLC-MS analyses of PADPA & D5-aniline (**Fig. S-6b**) and PADPA & D1-aniline (**Fig. S-6c**) reaction mixtures were carried out in order to determine the exact structure of the aniline trimer. These experiments show that an amino group of PADPA forms a bond to the C4 carbon of aniline. Two types of trimers can be formed this way: linear (bond formation with the primary amino group of PADPA) or branched (bond formation with the secondary amino group of PADPA) (**Scheme S-3**). In order to resolve which of these two possible structures formed, classically synthesized aniline trimer (**Scheme S-4** and **Fig. S-7**) was utilized as reference compound. It was treated, separated and detected the same way as the samples from the reaction mixtures, and was found to have the same retention time and spectrum as the trimer formed in the reaction (**Fig. S-8**). Thus, the aniline trimer formed during oligomerization is linear, N−C-*para* coupled and is obtained *via* bond formation between the primary amino group of PADPA and the C4 carbon of aniline. This coupling is shown for the PADPA & D1-aniline mixture in **Scheme 2**, without taking into account the actual oxidation or protonation states of PADPA, aniline or aniline trimer. The primary amino group of PADPA is marked bold and in blue color. The deuterium atom of the D1-aniline is lost upon *p*-coupling. We propose that a partially oxidized radical cation form of the linear aniline trimer is responsible for the intensive band at 700 nm in the *in situ* recorded UV/vis/NIR spectra due to the similarities in time dependent changes of A_{700} (**Fig. 2b**) and those of the chromatographic peak at rt ≈ 30 min (**Fig. 4b**). Further, the

absorption band at ≈700 nm observed during reaction resembles the one reported for a related oligomer, the paramagnetic radical cation form of the phenyl end-capped aniline dimer ($B_3^{\bullet+}$),

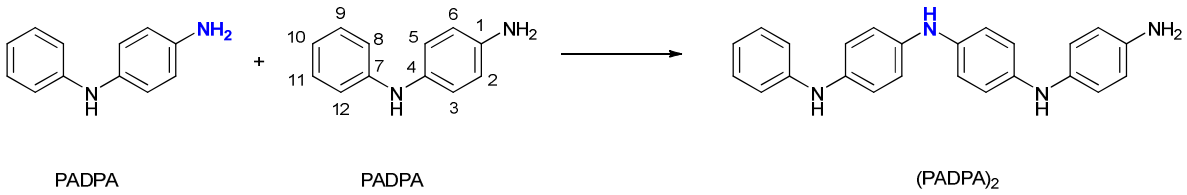
see section 3.3.[43,44] **Figure 4c** shows that a substantial amount of trimer is still left over after 24 h, although the band at 700 nm has decreased completely (**Fig. 2a**). This indicates that the linear N−C-*para* coupled aniline trimer intermediate becomes oxidized during this phase of the reaction (8 min → 24 h). The fully reduced and deprotonated aniline trimer has an absorption maximum at 302 nm (**Fig. S-9**).



Scheme 2: Simplified drawing of the formation of the linear N−C-*para* coupled aniline trimer from PADPA and D1-aniline, *without* taking into account protonation or oxidation states. The loss of the deuterium atom from D1-aniline during the formation of the aniline trimer is evident from the determined mass of the aniline trimer (m/z, z = 1). Found: 276.1495; calculated: 276.1495; calculated for an aniline trimer with one deuterium atom: 277.1558.

The next prominent reaction product elutes at rt ≈ 35 min (**Fig. 4a**). At the beginning, its quantity is quite low, but steadily increases until it is one of the major products after 3 days (peak 4 in **Fig. 4a** and **Fig. 4c**). This peak stems from the linear, N−C-*para* coupled PADPA dimer, $(PADPA)_2$ (linear tetraaniline, **Fig. S-9**), which we propose to be in the protonated, emeraldine salt form in the reaction suspension. It is primarily responsible for the absorption at ≈1000 and ≈420 nm in the UV/vis/NIR spectrum of the reaction mixture after 1-3 days (**Fig. 1a**).[36]

$(PADPA)_2$ is likely obtained by a bond formation between the primary amino group of a PADPA molecule and C10 of another PADPA molecule (head-to-tail coupling). This is shown in **Scheme 3**, without taking into account oxidation or protonation states. The reaction seems to occur steadily, but quite slowly *via* the highly reactive intermediate *N*-phenyl-1,4-benzoquinonediimine (PQD).[36] The yield of $(PADPA)_2$ after 24 h in the mixed PADPA & aniline system is higher than in the reaction mixture with PADPA only (**Fig. 4c**).[33] The reason for this difference is not clear. It could be that part of the $(PADPA)_2$ molecules are obtained from the coupling of the aniline trimer with aniline.



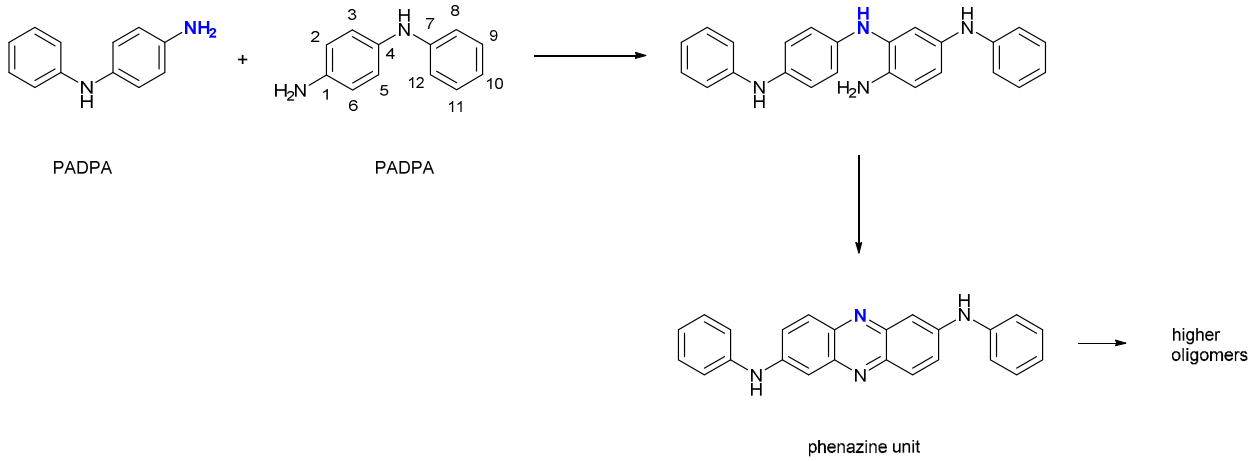
Scheme 3: Formation of the linear PADPA dimer from two PADPAs without taking into account the protonation or oxidation states of the molecules.

The peak in the chromatograms at rt ≈ 40 min is broad and ill-defined (**Fig. 4a** and **Fig. 4c**). It stems from a linear, N–C-*para* coupled aniline hexamer (**Fig. S-9**). The structure determination was not straightforward because this material was not completely reduced after treatment with hydrazine; different oxidation states of the aniline hexamer could be identified in the mass spectrum. Nevertheless, the integral of the peak at rt ≈ 40 min steadily increases during the reaction until it is the product that yields the largest integral in the chromatograms (see **Fig. 4b**). Since the absorption spectra of the different oligoanilines are expected to have different molar

absorptions at 310 nm ($\pi \rightarrow \pi^*$ transition),[46,47] a direct comparison of the integrals of aniline trimer, tetramer and hexamer is not possible. Nevertheless, there is no doubt that aniline hexamers form in the reaction mixture of PADPA & aniline, as it was found for the reaction with PADPA alone (**Fig. 4c**).[33] The aniline hexamer could either form *via* the addition of a second PADPA molecule to $(PADPA)_2$ or from two aniline trimers. Interestingly, there is no evidence for the formation of an aniline pentamer. Its rapid increase in the beginning followed by a slow decrease with time (**Fig. 4b**) suggests that dimerization of the aniline trimer is the dominating reaction in the formation of the aniline hexamer. If the integral of the broad peak at rt ≈ 40 min for the reaction of PADPA & aniline is compared to the one for the reaction of PADPA alone (**Fig. 4c**), the higher extent of aniline hexamer formation in the monomer mixture over PADPA alone is obvious after 2 h. Aniline hexamer may contribute to the *in situ* UV/vis/NIR signal at ≈1000 and ≈420 nm (**Fig. 1a**), as well as to the strong EPR signal (**Fig. 1b**).

Finally, there are a number of peaks with retention times from 55 to 65 min. Generally, these peaks stem from aniline or PADPA oligomers, $(aniline)_4$ to $(aniline)_{10}$ that have a phenazine core. This assignment is based on their UV/vis spectra with their secondary absorption maxima at ≈500 nm and on their masses. As mentioned above in section 3.2. and discussed previously,[33] we propose that products containing phenazine units are responsible for the *in situ* observed bands at ≈500 nm. We hypothesize that the initial phenazine-type oligomer is a PADPA dimer and is formed via a bond formation between a primary amino group of one PADPA molecule to a C2 or C6 atom of a second PADPA molecule (head-to-head attack), followed by cyclization resulting in a PADPA dimer with a phenazine core (**Scheme 4**),[48] which appears as sharp peak with rt ≈ 51 min, after a reaction time of 0.5 min (**Fig. 4a**). The addition of another PADPA molecule leads to $(PADPA)_3$ which elutes at rt ≈ 56 min (**Fig. 4a**) and of which the structure is

not known. Continued addition of aniline or PADPA yields longer oligomers with peaks with at rt > 56 min. However, structures and identities of these higher oligomeric species are not clear, especially because not only PADPA, but also aniline may be involved in their formation. Oligomers with phenazine units in a PANI-ES type material are generally considered as unwanted side products, which neither contribute to the absorption in the NIR at ≈1000 nm nor to the EPR signal. The direct comparison of the chromatograms for the PADPA & aniline reaction mixture and for the reaction of PADPA only − both reactions run for 24 h under the corresponding optimal conditions – reveals that the products obtained from the PADPA & aniline mixture contain smaller amounts of oligomers with phenazine motif (**Fig. 4c**, rt = 55-65 min). This is in full agreement with the Raman spectroscopy measurements (**Fig. 3**).



Scheme 4: Suggested formation of a PADPA dimer with a phenazine core, from which longer oligomers may grow.

3.5. Cyclic Voltammetry (CV) Measurements.

For the CV measurements, the as obtained vesicle suspension containing the PANI-ES-like products resulting from the reaction of the PADPA & aniline mixture was deposited on a glassy carbon electrode (see 2.6.) and then analyzed for its redox activity, see **Figure 5** (trace 1). Comparison was made with a reaction mixture obtained from PADPA alone (**Fig. 5**, trace 2). From **Figure 5** it is evident that the PANI-ES-like reaction products obtained from the PADPA & aniline mixture are significantly better at charge transfer than the PANI-ES-like oligo(PADPA) products obtained from PADPA alone. This finding is consistent with the *in situ* UV/vis/NIR, *in situ* EPR, and *in situ* Raman spectroscopy measurements, see above.

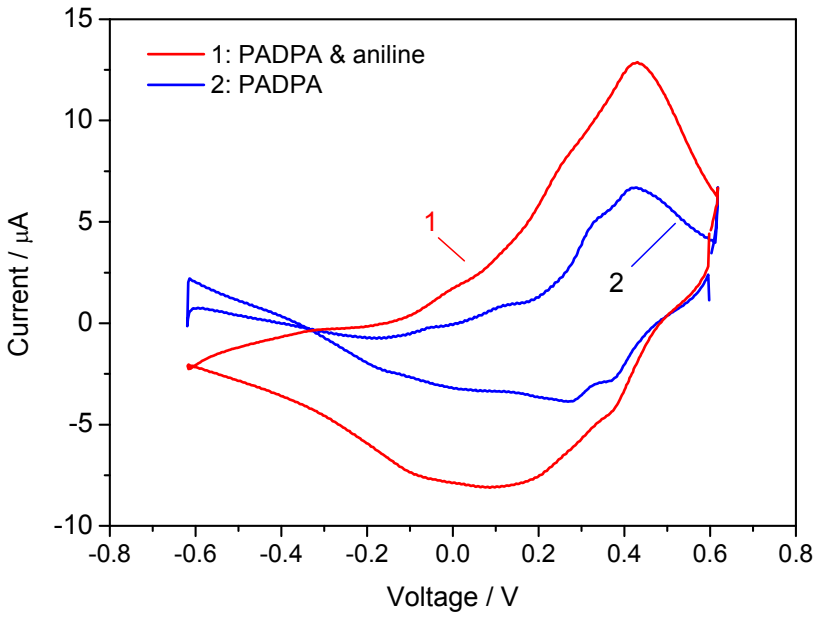


Figure 5. Cyclic voltammograms of the products obtained from the PADPA & aniline mixture (1, red) and from PADPA alone (2, blue) with HRP/H_2O_2 in the presence of AOT vesicles under their optimal conditions after 24 h (see the legend of **Fig. 1**). The as-obtained vesicle suspensions were deposited on a glassy carbon electrode. Scan rate: 100 mV/s.

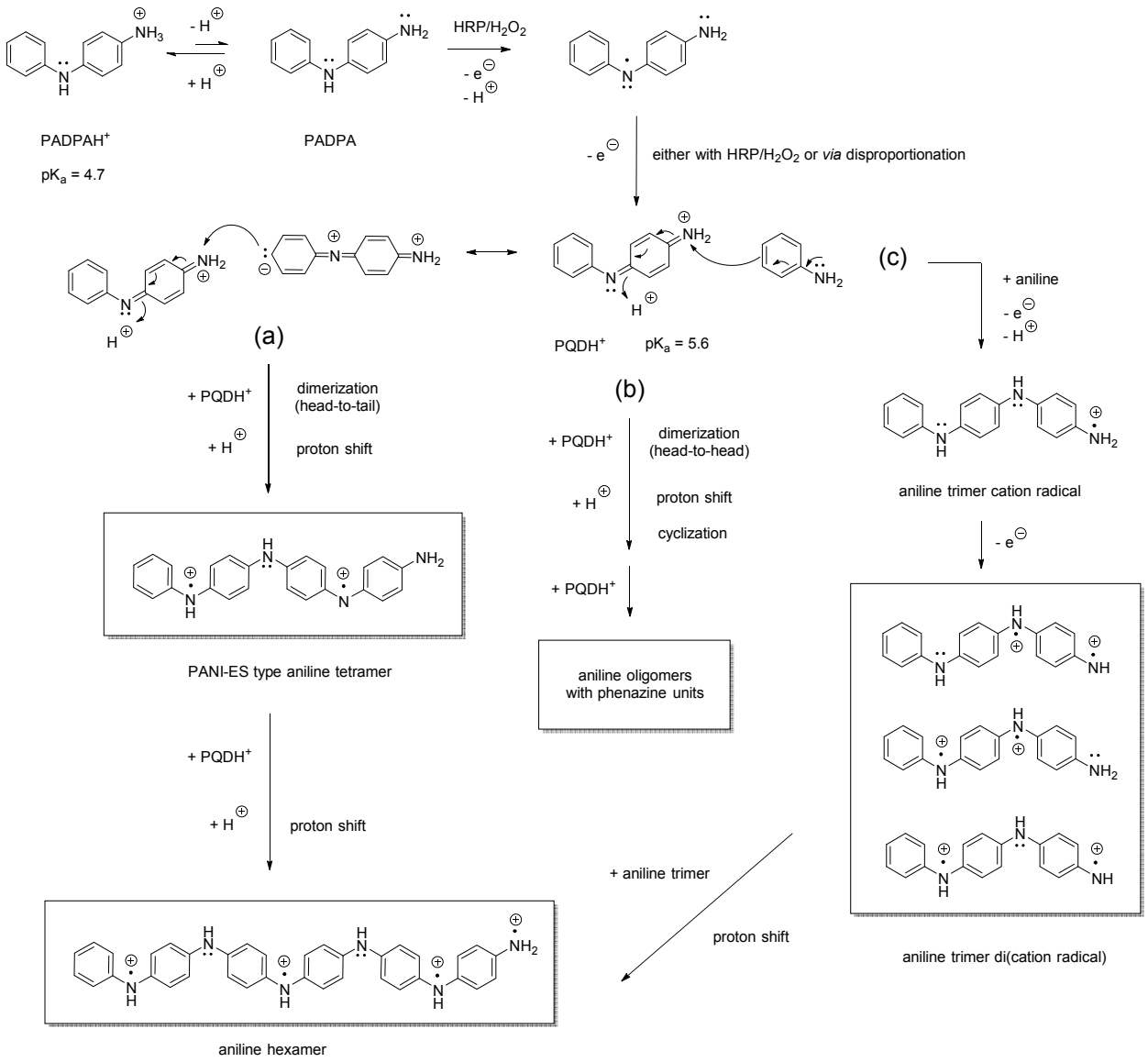
3. 6. Reaction Scheme Proposal

Based on the preceding discussion (see 3.4.); on the fact that for the conditions used in this work, the direct oxidation of aniline with HRP/H_2O_2 is negligible (**Fig. 1a**); and our previous work on the reaction of PADPA with either HRP/H_2O_2 or TvL/O_2,[33,36] we propose the following reaction scheme for the reaction of PADPA & aniline with HRP/H_2O_2 in the presence of AOT vesicles. PADPA is involved in (at least) three different reaction routes. In all cases, PADPA is first oxidized by the peroxidase to the PADPA radical, which is then oxidized again, either by

the enzyme or by disproportionation, to PQD (*N*-phenyl-1,4-benzoquinonediimine), which, at pH = 4.3, is most likely extensively protonated ($PQDH^+$, $pK_a(PQDH^+) = 5.6$).[49] This highly reactive species can then undergo the coupling reactions illustrated in **Scheme 5**. Reaction *route (a)* shows the head-to-tail coupling of two $PQDH^+$, which, after proton shifts and internal rearrangement of the electrons, forms $(PADPA)_2$ in the emeraldine salt form with separated polarons.[36] This molecule is quite stable and unreactive and, as discussed above, most likely does not undergo further reactions, besides, a possible chain growth to the aniline hexamer, but only to a small degree. Reaction *route (b)* is the head-to-head coupling of two $PQDH^+$ cations to yield aniline oligomers with a phenazine core. This route is more prominent in the reaction of only PADPA with HRP[33] than in the reaction of PADPA & aniline with HRP. To what extent the aniline molecule is involved in *route (b)* is not clear. Finally, reaction *route (c)* shows the reaction between $PQDH^+$ and aniline. In comparison to the previous systems we have studied, reaction *route (c)* is new. We propose the following mechanism: as with the other two routes, PADPA is first oxidized to $PQDH^+$ while aniline does not undergo substantial oxidation by the enzyme. This assumption is based on the fact that PADPA is much easier to oxidize than aniline, and also based on the *in situ* UV/vis/NIR spectrum of aniline only and HRP/H_2O_2 (**Fig. 1a**, spectrum 3) where it is shown that by using such a low concentration of HRP, aniline is not oxidized.[31] As a next step, there must be a bond formation between the primary amino group of PADPA and the C4 atom of aniline. As is shown in **Scheme 5**, the mechanism for this bond formation may be a simple aromatic electrophilic addition of $PQDH^+$ to aniline, where the amino group of the aniline is *para*-directing. Thus, the aniline trimer in the oxidized (reactive) form is obtained. The fully reduced aniline trimer does not absorb in the visible range, but the *in situ* UV/vis/NIR spectra (**Fig. 2a**) show a sharp band at 700 nm. A band at 700 nm is consistent with

the formation of an oxidized radical cation form of the aniline trimer as shown in **Scheme 5**.[50] This form is still reactive and (i) may either combine with another trimer to form an aniline hexamer; or (ii) may convert into the aniline trimer in its reduced form. The slow electrophilic aromatic addition between two aniline trimers – case (i) – may yield a linear aniline hexamer. Furthermore, the addition of one aniline molecule to form the aniline tetramer is also possible. Now that the aniline chain is longer, it can undergo rearrangement and aromatization to form the aniline hexamer with separated polarons as PANI-ES which absorbs in the NIR.

Of course, due to the complexity of the reaction, other reaction routes are also feasible. However, it is proposed that the routes discussed in **Scheme 5** are the prominent ones.



Scheme 5: Suggested key reactions which take place in the AOT vesicle-assisted reaction of the PADPA & aniline mixture with HRP as catalyst and H_2O_2 as oxidant for the conditions used (see the legend of **Fig. 1**). The initial two one-electron oxidations of PADPA yield – after protonation – $PQDH^+$, the protonated form of PQD (*N*-phenyl-1,4-benzoquinonediimine). This reaction intermediate may undergo three different follow-up reactions, (a)-(c), yielding the aniline tetramer in the PANI-ES-like state, $(PADPA)_2^{2(\bullet+)}$, aniline oligomers with undesired phenazine units, or the aniline trimer, which further reacts to the aniline hexamer.

4. CONCLUSIONS

The enzymatic preparation of processable aqueous conductive PANI-ES solutions or suspensions with *high conversion* and by using *low amounts of enzyme* is one of the scientific challenges we have set for ourselves for several years to develop a sustainable, environmentally-friendly, "green" polyaniline synthesis process. With aniline as starting material, the PANI-ES products obtained lead to severe enzyme inactivation such that the use of high amounts of enzyme is unavoidable for high conversions, as shown for example in the case of HRP and AOT vesicles as "templates".[31] Considerable progress in solving this "enzyme concentration problem" was the use of the linear aniline dimer PADPA instead of aniline.[33-36] With AOT vesicles and the elaborated optimal conditions, the amount of HRP[33] or TvL[34-36] could be reduced significantly, while still keeping the conversion high (>95%). With TvL/O_2, the PANI-ES-like products obtained from PADPA have many characteristic properties of ideal PANI-ES,[34-36] although it became clear that the products were "only" oligomers (mainly the PADPA dimer, *i.e.,* tetraaniline) and not true polymer molecules. The situation with HRP/H_2O_2 was found to be similar, *i.e.,* the HRP concentration could also be reduced significantly, and $(PADPA)_2$-ES was one of the main products.[33] However, with HRP/H_2O_2, unwanted hydrolysis reactions and the formation of substantial amounts of products which contained undesired phenazine units could not be avoided.[33] The reason for this is not clear but seems to be a direct consequence of the difference between the two enzyme/oxidant systems, which operate mechanistically in completely different ways, although in both cases PADPA is initially oxidized in a one-electron oxidation, HRP/H_2O_2, pH = 4.3, *vs.* TvL/O_2, pH = 3.5.

In the work presented here, we have shown that the HRP/H_2O_2-PADPA system can be improved substantially by using a PADPA & aniline mixture instead of PADPA only. The amount of HRP that can be used (30 nM) is still low for the efficient conversion of 2.0 mM aniline units (0.7 mM PADPA + 0.6 mM aniline) with 1.3 mM H_2O_2. And, most importantly, the resulting AOT vesicle suspension containing the PANI-ES-like products is not only stable but the PANI-ES-like products were found to be considerably more electroactive than the PANI-ES-like oligo(PADPA) products obtained from PADPA only (**Fig. 5**). All three *in situ* spectroscopic methods used, Uv/vis/NIR (**Fig. 1a, Fig. 2a,b**), EPR (**Fig. 1b, Fig. 2b**), and Raman (**Fig. 3**), agreed with each other and provided complementary information about the products obtained, in particular on their oxidation and protonation states, which are essential for their properties and direct use. Only the half oxidized, protonated form of linear poly- or oligoaniline is the electrically conductive one.[25,26] With the *ex situ* HPLC-DAD and HPLC-MS analysis it was shown that the product distribution was different from the reaction with PADPA only (**Fig. 4**). The aniline trimer was found to be not only a prominent reaction intermediate but it was also still present to a substantial amount after completion of the reaction (**Fig. 4c**, peak 3), together with the aniline tetramer (**Fig. 4c**, peak 4) and the aniline hexamer. Based on (i) the reported paramagnetic properties of a doped trimeric aniline derivative carrying four amino groups as redox centers and with absorption at ≈1000 nm in chloroform solution,[51] and based on (ii) the absorption spectrum and paramagnetic behavior of a doped phenyl end-capped aniline dimer,[43] one is tempted to conclude, that the aniline trimer (in its radical cation-polaron form) which formed in the PADPA & aniline reaction mixture contributed to the EPR signal and is mainly responsible for the absorption at ≈400 nm and ≈700 nm in the visible region of the spectra. However, the previously mentioned trimeric aniline derivative shows very low absorbance

around 400 nm,[51] which is completely different from the spectroscopic properties of the reaction products we obtain from PADPA & aniline (**Fig. 1a**). Moreover, the repeating unit of ideal PANI-ES is known to be the aniline tetramer (**Scheme 1**). Therefore, the increased redox activity, EPR signal intensity, values of $A_{\approx 1000}$ and $A_{\approx 400}$, and intensity of $\nu(C\sim N^{\bullet+})$ Raman band at about 1340 cm^{-1} of the reaction products obtained from the PADPA & aniline mixture at later stages of the reaction are most likely due to the decreased extent of formation of products with phenazine units (**Fig. 4c**, rt = 55-65 min), the increased amounts of $(PADPA)_2$ in its delocalized polaron form with increased chain linearity and ring coplanarity (extended coil conformation) (**Fig. 4c**, peak 4), and the absence of oxygen atom-containing products.

Tetraaniline in its delocalized polaron form of the emeraldine salt, $(PADPA)_2^{2(\bullet+)}$, is of particular interest for fundamental studies of model compounds of PANI-ES and for possible applications, as recently demonstrated for a chemically synthesized product.[24,52-55] With our enzymatic method using AOT vesicles as templates, $(PADPA)_2^{2(\bullet+)}$ is obtained as one of the main products (**Fig. 4c**, peak 4),[34,36] and the as-obtained suspension is stable and can be applied without the need of further treatments.[33,35] This is an important issue for intended practical applications.[56,57] The conductive, paramagnetic $(PADPA)_2^{2(\bullet+)}$ aniline tetramer with delocalized polarons explains the appearance/strengthening of 'polaron bands' observed at 1140 nm in the UV/Vis/NIR spectrum and at ≈1340 cm^{-1} in the Raman spectrum in later stages of the reaction.

Along a more general line, we would like to recall with our detailed study that in complex reaction mixtures – similarly to biological systems – the results provided by the reacting molecules, *i.e.,* the outcome of a reaction, strongly depends on how the reaction is triggered, *i.e.,* which components are mixed at which concentrations, in which medium, in which order, at

which temperature, and for how long the mixture is left reacting. The more complex the reaction mixture is, the more it becomes challenging not to miss parameter combinations that yield desired results, if these can be obtained at all. For the systems we investigate, the target properties of the products are relatively clear. They need to have the characteristic properties of electrically conductive, paramagnetic PANI-ES with expected absorption bands at ≈1000 nm and ≈420 nm,[25,26] and low absorption at ≈600 nm.[33] This allows a relatively straightforward screening for finding optimal reaction conditions. Nevertheless, the complex nature of the reaction, involving on the one hand radicals, which undergo rapid reactions, and on the other hand radicals which are stabilized, *i.e.,* not reactive, makes the entire reaction optimization still a big challenge.

In a follow-up work on the enzymatic synthesis of PANI-ES-like products, we still like to improve our understanding of the role of the "template" (the reaction medium) by making a direct comparison of the outcome of the reaction in dependence of the presence of anionic vesicles *vs.* anionic micelles *vs.* anionic polyelectrolytes, by using experimental conditions which can be compared. The final goal is the application of the as-obtained products, for example for the development of biosensor devices.[58] The first steps have been made by showing the possibility of coating electrodes,[33,35,59] or for using the vesicle suspensions as ink in a conventional inkjet printer.[32]

ASSOCIATED CONTENT

Supporting Information. The Supporting Information file (PDF) is available free of charge and contains clarifications about the chemical structures and reactions discussed; experimental data

on the influence of the ratio of PADPA to aniline and of the HRP concentration on the outcome of the reaction; deconvolution of Raman spectra; quantification of remaining PADPA and aniline; and additional HPLC-MS analyses.

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