The Influence of Anionic Vesicles on the Oligomerization of *p*-Aminodiphenylamine Catalyzed by Horseradish Peroxidase and Hydrogen Peroxide

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Abstract

The aniline dimer *N*-phenyl-1,4-phenylenediamine (= *p*-aminodiphenylamine, PADPA) was oxidized with horseradish peroxidase isoenzyme C (HRPC) and hydrogen peroxide (H₂O₂) to oligo(PADPA) in an aqueous suspension of 80-100 nm-sized anionic vesicles at pH = 4.3 and at T ≈ 25 °C. The vesicles were formed from AOT (= sodium bis(2-ethylhexyl) sulfosuccinate) and served as templates for obtaining oligo(PADPA) as emeraldine salt form of polyaniline (PANI-ES) in the polaron form. The optimal reaction conditions for obtaining a stable oligo(PADPA)-AOT vesicle suspension with a high conversion and low amounts of HRPC were elaborated by using UV/vis/NIR spectroscopy. The formation of PANI-ES type products was confirmed by *in situ* UV/vis/NIR, Raman and EPR spectroscopy measurements. However, HPLC-MS analyses indicated that the oligo(PADPA) products obtained are not

only of PANI-ES type, but that a number of other products formed as well. Some of these were identified and shown to contain oxygen atoms (as a result of hydrolysis), others most likely containing phenazine (or phenoxazine) moieties. The latter is also supported by Raman and UV/vis/NIR spectroscopy measurements. The as-obtained oligo(PADPA)-AOT vesicle suspension was also analyzed by cyclic voltammetry, which showed redox activity of oligo(PADPA) up to pH = 6.0, a property which is beneficial for various applications. Overall, the work demonstrates the successful use of different complementary and sensitive *in situ* spectroscopic methods for following the progress of complex enzyme-triggered reaction. In addition, the good solubility of the deprotonated and reduced reaction products in organic solvents allowed us to use a HPLC-MS analysis for a direct comparison of the product distribution with the previously studied oxidation and oligomerization of PADPA with *Trametes versicolor* laccase at pH = 3.5. One of the template effects of AOT vesicles takes into account possible interactions between AOT and oligo(PADPA).

Keywords: enzymes, oxidoreductases, polymers, template synthesis, vesicles

1. Introduction

From a number of previous studies it has become clear that aqueous suspensions of anionic vesicles with a diameter of about 80-100 nm are suitable reaction media for the enzymatic oligo- or polymerization of aniline [1-6], the aniline dimer *N*-phenyl-1,4-phenylenediamine (= *p*-aminodiphenylamine = PADPA; the trivial name is *p*-semidine) [7,8], or pyrrole [9]. With “suitable” we mean (i) that the presence of the vesicles as templates has a positive and desired influence on the outcome of the reaction with respect to the chemical structure of the products formed [10], (ii) that the reaction occurs with a high monomer

conversion, and (iii) that no significant product precipitation is observed. Through an optimization of the reaction conditions in the case of aniline [1–6] or PADPA [7, 8], reaction products can be obtained which resemble the conductive emeraldine salt form of polyaniline, PANI-ES, in its polaron state (with unpaired electrons and high absorption in the near infrared, NIR). This is very similar to the enzymatic polymerization of aniline with other types of macro- or polymolecular templates, *i.e.* anionic polymers [10–17] or anionic micelles [10,13,17–20]. For the vesicular templates investigated, those formed from AOT (= sodium bis(2-ethylhexyl) sulfosuccinate) were found to be particularly suitable [2–9]. The enzymes used so far for the polymerization of aniline in the presence of AOT vesicles include horseradish peroxidase isoenzyme C with hydrogen peroxide as oxidant (HRPC/H₂O₂) at pH = 4.3 [2–4] and *Trametes versicolor* laccase with dissolved dioxygen as oxidant (TvL)/O₂ at pH = 3.5 [5,6]. However, there are three main disadvantages associated with using *aniline* as monomer: (i) generally, a high amount of enzyme is needed (0.1 g HRPC required for the conversion of 1.0 g aniline, [3]); (ii) slow reaction and the formation of over-oxidized products with TvL/O₂ [5]; and (iii) the final product mixture is not extractable and can therefore not be analyzed in detail. Using PADPA as monomer instead of aniline and TvL/O₂ as catalyst/oxidant resolves all three issues [7]. Concerning the amount of enzyme required for the conversion of 1.0 mM PADPA into oligo(PADPA) products, only ≈ 32 nM TvL are needed [7], corresponding to ≈ 10 mg pure TvL for 1.0 g PADPA, as calculated by taking into account $M(\text{PADPA}) = 184.2$ g/mol and $M(\text{TvL}) = 66000$ g/mol [21]. The oxidation of PADPA with TvL/O₂ in the presence of AOT vesicles was recently investigated in great detail by *in situ* UV/vis/NIR, EPR, and Raman spectroscopy measurements [8], as well as product analysis by HPLC separation and detection with a diode array UV/vis and a MS detector [22]. The latter studies have shown that with AOT vesicles, the main oligo(PADPA) product formed with TvL/O₂ is the PADPA dimer, which is linear, C~N coupled and the shortest

aniline oligomer which can exhibit PANI-ES spectroscopic characteristics [22]. It was found that this PADPA dimer has a different distribution of unpaired electrons than ordinary PANI-ES [23]. Another reaction route in the presence of AOT vesicles leads to the formation of oligomeric, phenazine-containing species [8,22].

In the work carried out by Shumakovich et al [24], PADPA was oxidized and oligomerized with *Trametes hirsuta* laccase in the presence of SDBS micelles as templates at $pH = 3.8$. Also in that work, the formation of aniline oligomers was reported [24].

The motivation behind the work presented now was to determine whether PADPA can also be oligomerized efficiently with HRPC/H₂O₂ to PANI-ES type products by using AOT vesicles as “chemical structure-controlling templates” [10], like in the case of TvL/O₂ [7,8,22]. Since this was found to be the case, we questioned whether there exist significant differences between the products obtained under optimal reaction conditions with HRPC/H₂O₂ and the products obtained with TvL/O₂ [7,8,22], and if yes, what these differences are. For a direct comparison of the two systems, the analytical methods applied previously for analyzing the system with TvL/O₂ [7,8,22] were also used for the reaction with HRPC/H₂O₂.

2. Experimental

2.1. Chemicals

AOT (= sodium bis-(2-ethylhexyl) sulfosuccinate), ABTS²⁻ (= 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) and PADPA (= *p*-aminodiphenylamine = *N*-phenyl-1,4-phenylenediamine) were purchased from Sigma-Aldrich. HRPC (= horseradish peroxidase isoenzyme C, product PEO-131, grade I, lot 2131616000,

287 U/mg) was from Toyobo Enzymes. A “pH = 4.3 solution” was prepared from deionized water, NaH₂PO₄ (from Sigma-Aldrich, ≥ 99%) and a 1 M solution of H₃PO₄ (made from a 85 wt% solution from Fluka) to yield a total concentration of 0.1 M (= [H₂PO₄⁻ (aq)] + [H₃PO₄ (aq)]) and a pH value of 4.3.

2.2. Stock solutions and AOT vesicle suspension

The PADPA in “pH = 4.3 solution” (1.5 mM), H₂O₂ in milli-Q water (200 mM) and a AOT (20 mM) in “pH = 4.3 solution” vesicle suspension were prepared as described previously [2,3,7]. HRPC stock solution I with [HRPC] = 67 μM was prepared by dissolving 3.89 mg/ml HRPC in the “pH = 4.3 solution”. The exact concentration was determined spectrophotometrically with $\epsilon_{403\text{ nm}}(\text{HRPC}) = 1.02 \cdot 10^5 \text{ M}^{-1}\text{cm}^{-1}$ [25]. HRPC stock solution II was made by 100 x dilution of HRPC stock solution I to yield [HRPC] = 0.67 μM, which was used for the reaction.

2.3. Reaction of PADPA with HRPC/H₂O₂ in the presence and absence of AOT vesicles

The reaction was carried out at room temperature ($T = 22\text{--}25\text{ }^{\circ}\text{C}$) either in 2 mL polypropylene Eppendorf tubes[®] with a total reaction volume, V_r , of 0.5 mL, or in 50 mL Schott Duran[®] laboratory glass bottles with $V_r = 10\text{ mL}$. For the elaborated optimal conditions, the reaction mixture was prepared in the following way ($V_r = 10\text{ mL}$): 1.84 mL “pH = 4.3 solution” was first added to the reaction vessel, then 1 mL AOT vesicle suspension, then 6.67 mL PADPA solution, then 448 μL HRPC stock solution II, and finally 50 μL H₂O₂ solution. For $V_r = 0.5\text{ mL}$, the volumes of the different solutions were adjusted accordingly. The concentrations in this optimal reaction mixture were: 2.0 mM AOT, 1.0 mM PADPA, 30 nM HRPC, 1.0 mM H₂O₂, $pH = 4.3$ (0.1 M H₂PO₄⁻), see **Scheme 1**. For the reactions without vesicles (template-free system), the “pH = 4.3 solution” was used instead of the vesicle suspension.

2.4. Characterization methods

UV/vis/NIR, EPR, and *in situ* Raman spectroscopy measurements were conducted as described in detail in our previous publication on the analysis of the reaction of PADPA with TvL/O₂ [8]. Despite the slightly different reaction system, the equipment and the analytical strategy remained the same. The Raman spectra were recorded with the same reaction mixture (with or without vesicles), without interrupting the reaction (*in situ*), by withdrawing 5 μ L aliquots of the reaction mixture from the reaction vessel at specified reaction times and transferring them into sample wells at the sample slide (more details are given in Supplementary information of ref. [8]). All the Raman spectra are shown after automated fluorescence correction performed by the OMNIC software (Thermo Scientific). For the UV/vis/NIR measurements at the early stage of the reaction, a diode array spectrometer Specord S 600 from Analytik Jena AG was used with Hellma quartz cells of 0.1 cm path length. The activity of HRPC in the reaction mixtures was determined spectrophotometrically with ABTS²⁻ as substrate and H₂O₂ as oxidant, as described previously by Junker *et al.* [3]. The synthesis of penta-deuterated PADPA (D5-PADPA) and monodeuterated PADPA (D1-PADPA) as well as the HPLC-DAD (diode array UV/vis detection) and HPLC-MS measurements of the products of the reactions were conducted as described in the recent publication on the detailed structure elucidation of the products of the oxidation of PADPA with TvL/O₂ [22]. For cyclic voltammetry measurements, the reaction mixture was deposited on a glassy carbon (GC) disk electrode (5 mm diameter) by the direct drop-casting method followed by drying with a weak N₂ gas stream and then analyzed by cyclic voltammetry [8]. This approach was chosen because it suppresses modifications of the product due to isolation and purification and significantly simplifies the procedure [8].

3. Results and Discussion

3.1. *In situ* spectroscopic monitoring of the oxidation and oligomerization of PADPA with HRPC/H₂O₂

3.1.1. *Optimal reaction conditions*

The aniline dimer PADPA can be oxidized in aqueous solution by HRPC and H₂O₂, whereby the presence of AOT vesicles has a clear influence on the outcome of the reaction. Molecules of PADPA (the pK_a of the protonated form is 4.70 [26]) bind to the negatively charged vesicle surface and are oxidized by HRPC (isoelectric point 8.8 [27]), which itself is activated by H₂O₂, see **Scheme 1**. Formed PADPA radical cations (PADPA^{•+}) participate in subsequent coupling reactions leading to the formation of oligo(PADPA), which is a mixture of oligomeric and/or polymeric products, **Scheme 1**. Compared to the reaction of aniline [3], the amount of HRPC required is significantly lower (see below). The reaction products obtained are colored, and depending on whether AOT vesicles are present or not, the products either precipitate during the course of the reaction and turn red (no vesicles), or they remain finely dispersed without precipitation and turn green (with vesicles). In order to reach the highest possible conversion efficiency, colloidal stability and yield, the optimal conditions for the reaction with AOT vesicles were found by systematically varying the reaction conditions. The following initial composition turned out to be suitable for obtaining a stable dark green suspension, which looks at first sight like a suspension containing PANI-ES as desired product: 0.1 M H₂PO₄⁻ (pH = 4.3), 2.0 mM AOT, 1.0 mM PADPA, 30 nM HRPC, 1.0 mM H₂O₂, *T* ≈ 25 °C (room temperature), see **Scheme 1**. The reaction was carried out by first adding a PADPA solution (pH = 4.3) to an AOT vesicle suspension (pH = 4.3), then adding the HRPC stock solution II (0.67 μM), and finally a H₂O₂ solution to trigger the reaction (see Experimental Part for details).

3.1.2. In situ UV/vis/NIR absorption measurements

In **Figure 1**, the UV/vis/NIR spectra of the reaction with vesicles (1) and without vesicles (2) after 24 h are shown. With AOT vesicles, products are obtained which have a high absorption in the NIR region, at ≈ 1000 nm, a narrow band at 415 nm, and an unexpected band at ≈ 600 nm (atypical for PANI-ES). Control reaction mixtures containing either (i) PADPA, AOT vesicles, and HRPC, but no H_2O_2 , or (ii) PADPA, AOT vesicles, and H_2O_2 , but no HRPC, did not lead to significant changes in the UV/vis/NIR absorption spectrum after 24 h incubation. This indicates that catalyst and oxidant, HRPC and H_2O_2 , are required for the reaction to occur. Spectrum (1) in **Figure 1** is similar to the spectrum of the oxidation of PADPA with TvL/O_2 in the presence of AOT vesicles [7,8,22], with one big difference: when using TvL/O_2 , the band at ≈ 600 nm is not obtained. Therefore, the product distribution of the reaction of PADPA with HRPC/ H_2O_2 at $\text{pH} = 4.3$ must be different from that obtained with TvL/O_2 at $\text{pH} = 3.5$. It is obvious that the type of catalyst and oxidant used, and perhaps the pH , have a clear influence on the obtained oligo(PADPA) products. The bands observed at around 1000 nm and at 415 nm correspond to the polaron form of linear PANI-ES-like oligo(PADPA) chains [3,28]. The shortest oligomer of such a type is the linear $\text{N}_{\text{prim}}\text{-C}_{10}$ coupled PADPA dimer in the half-oxidized ES polaron-like form (structure **E**, **Scheme 2**). This aniline tetramer has indeed been found to be the main product of the reaction of PADPA with TvL/O_2 [22]. Therefore, it can be assumed that it is also formed with HRPC/ H_2O_2 as one of the products. The band at ≈ 600 nm is not characteristic for ordinary PANI-ES-like structures. It can be tentatively attributed to relatively short PADPA oligomeric structures which contain substituted *N*-phenylphenazine units, based on the Raman measurements (see below). An example of such structures is $\text{N}_{\text{prim(B)}}\text{-C}_{10(\text{PADPA})}$ coupled PADPA trimer in the *N*-phenylphenazine form (structure **K**, **Scheme 2**) which can be formed by the reaction of branched $\text{N}_{\text{prim}}\text{-C}_3$ coupled PADPA dimer (structure **B**, **Scheme 2**) with PADPA, leading to

the formation of branched trimer (structure **H**, **Scheme 2**), which further transforms to *N*-phenylphenazine form **K** by the oxidation and subsequent intramolecular cyclization reaction. The support for this assignment is from the UV/vis spectra of aniline oligomers which contain *N*-phenylphenazine-type units: they display absorption maxima at similar wavelengths [29–31].

Figure 2 shows complex time-dependent changes in the UV/vis/NIR absorption of the reaction with vesicles. At the very early stage of the reaction (**Fig. 2a**), after 10 s, two bands appear: one at ≈ 500 nm and one at ≈ 740 nm. The peak at 285 nm stems from PADPA. During the next stage of the reaction, between 10 s and 120 s, (i) a new band is formed at 310 nm and (ii) the two initial bands undergo shifts with a concomitant intensity increase (the band at 500 nm undergoes a slight shift to 495 nm, and the band at 740 nm is shifted to 850 nm). In the next phase of the reaction (2 – 60 min, **Fig. 2b**), the band at 495 nm decreases, while bands at 413 nm and 1015 nm appear (spectrum 5 in **Fig. 2b**). Simultaneously, the intensity of the band at ≈ 310 nm decreases, and the position of the band maximum shifts from 310 nm to ≈ 300 nm (spectrum 5 in **Fig. 2c**). In the third phase (60 min – 4 h, **Fig. 2c**), further changes in the UV/vis/NIR spectrum are obvious, most notably a decrease of the absorption intensity at ≈ 850 nm to yield a spectrum (spectrum 9 in **Fig. 2c**) which is similar to the one recorded after 24 h (**Fig. 1**). There are no large spectral changes anymore between 4 h and 24 h. **Fig. 2d** is a plot of the time-dependent changes in absorbance at 1060, 850, 600, 415, and 300 nm occurring during the reaction, summarizing the preceding description. Despite the complexity of the reaction and of the observed spectral changes, the reaction is quite reproducible with respect to the spectral changes occurring during the reaction and with respect to the characteristics of the final spectrum. In contrast, complex spectral changes during the course of the oxidation of PADPA with TvL/O₂ instead of HRPC/H₂O₂ – both

times in the presence of AOT vesicles – do not occur [7,8], indicating that the two reaction mechanisms are very different.

For the reaction of PADPA with HRPC/H₂O₂ but *without vesicles*, a band appears at ≈ 490 nm after 20 s and slightly decreases in intensity during the next 100 s (**Fig. 3**). Later on precipitation occurs, rendering all later UV/vis spectra difficult to interpret. This illustrates the drastic difference between the reactions with and without vesicles.

3.1.3. *In situ EPR spectroscopy measurements*

After 24 h reaction time in the presence of vesicles, products with absorption in the NIR region are obtained (**Fig. 1**, curve 1), indicating the formation of products with unpaired electrons [28]. Indeed, there is also a strong EPR signal. The time dependent changes of the EPR spectrum in the presence of vesicles are shown in **Fig. 4**. There is a strong increase in radical concentration during the first 60 min of the reaction. *Without vesicles*, no EPR signal can be detected at least for the early phase of the reaction (**Fig. S1**).

In the case of the reaction of PADPA with TvL/O₂, products with unpaired electrons were obtained for both the reaction with and without vesicles, although in the latter case the radical concentration was considerably lower than in the system with AOT vesicles [7,8]. This direct comparison between the HRPC/H₂O₂ and the TvL/O₂ systems shows again that they are quite different and also indicates that the AOT vesicles have a different effect on the two systems. It seems that the PADPA radicals produced by HRPC/H₂O₂ in the absence of vesicles are short-lived and form products which do not have a noticeable amount of unpaired electrons.

3.1.4. *In situ* Raman spectroscopy measurements of the reaction in the presence of vesicles

The time-dependent changes of the *in situ* Raman spectrum of the reaction of PADPA with HRPC/H₂O₂ in the presence of AOT vesicles are shown in **Fig. 5** and **Fig. S2**. Each spectrum is complex and contains many bands which are tentatively assigned on the basis of literature [32–41], mainly related to PANI and oligo(PADPA) synthesized by classic oxidative polymerization. An overview of the main bands which were recorded and their assignments to functional groups/type of vibrations are presented in **Table 1**. In the following, the time-dependent changes of the different bands and corresponding functional groups are discussed; the analysis of the spectra is grouped according to the bands which are *typical* for PANI-ES and bands which are *atypical* for PANI-ES.

Bands typical for PANI-ES in the spectra of intermediates and products formed in the presence of vesicles:

There are a number of bands which are indicative of benzenoid (B), semiquinonoid (SQ) and quinonoid (Q) structural units, characteristic for the spectrum of PANI-ES (**Table 1**). Their relative intensities vary with reaction time and a number of them undergo a blue-shift during the reaction. For example, the intensity ratio of the $\nu(\text{C}\sim\text{C})_{\text{B}}$ band (1624 cm^{-1}) and the $\nu(\text{C}\sim\text{C})_{\text{SQ}} / \nu(\text{C}=\text{C})_{\text{Q}}$ band (1592 cm^{-1}) increases with time during the first ≈ 40 min, while the latter band undergoes a blue shift continuously during the reaction (1592 cm^{-1} at $t = 1\text{ min} \rightarrow 1599\text{ cm}^{-1}$ at $t = 19\text{ days}$). In addition, a new peak at $\approx 1610\text{ cm}^{-1}$, also attributable to $\nu(\text{C}\sim\text{C})_{\text{B}}$ vibrations, slowly develops. Its appearance indicates the formation of a new type of aromatic ring and a more complex structure of oligo(PADPA) products compared with PANI-ES. Its intensity steadily increases, and at longer reaction times it is comparable with the intensity of the adjacent peak at $\approx 1599\text{ cm}^{-1}$ (**Fig. S1**). Two further bands, indicative of SQ and B structures, undergo blue shifts during the reaction: the $\delta(\text{N-H})_{\text{SQ}}$ (1511 cm^{-1} at $t = 1$

min $\rightarrow$ 1518 cm^{-1} at $t = 19$ days) and $\delta(\text{C-H})_{\text{B}}/\delta(\text{C-H})_{\text{SQ}}$ (1179 cm^{-1} at $t = 1$ min $\rightarrow$ 1184 cm^{-1} at $t = 19$ days) bands. There are two $\nu(\text{C-N}^{++})_{\text{P}}$ bands, attributable to polaronic structures, P, which indicates that at least some of the oligo(PADPA) products have an oxidation and protonation state which is comparable to that of PANI-ES. One band is observed at 1315–1330 cm^{-1} ; a band at similar wavenumber range was found in the spectra of ordinary PANI-ES and was attributed to delocalized polarons [33,35]. The second $\nu(\text{C-N}^{++})_{\text{P}}$ band is observed at ≈ 1350 cm^{-1} and indicates a lower level of polaron delocalization in the products [33]. A contribution to this band from $\nu(\text{C-N}^{+})$ vibrations in substituted phenazine-type segments is possible [33]. The intensity ratio of the two bands at 1315–1330 cm^{-1} and ≈ 1350 cm^{-1} changes with time, indicating a gradual reorganization of the polaron structures. During the first ≈ 50 min the intensity of the band at ≈ 1320 cm^{-1} (related to the band at ≈ 1350 cm^{-1}) increases, indicating an increase of the content of more delocalized polaron structures (most probably in linear half-oxidized protonated structures such as **E** and **J** in **Scheme 2**). For later times, the peak at about ≈ 1350 cm^{-1} becomes more intense than that at ≈ 1330 cm^{-1} (**Fig. S2**). The relative intensity of both $\nu(\text{C-N}^{++})_{\text{P}}$ peaks increases during all the time of reaction, finally leading to a very strong band ($t=19$ days, **Fig. S2**), which indicates that the final oligo(PADPA) product mixture has good conductivity.

Bands atypical of PANI-ES in the spectra of intermediates and products formed in the presence of vesicles:

For the products of the PADPA oxidation/oligomerization with HRPC/ H_2O_2 in the presence of vesicles, a number of further bands which are not associated with PANI-ES were found, indicating the formation of other products, as was found previously to be the case for the TvL/ O_2 system [8]. These PANI-ES atypical bands mainly stem from phenazine-type structures, perhaps formed *via* intramolecular cyclization (**B** $\rightarrow$ **F** or **H** $\rightarrow$ **K** in **Scheme 2**).

Their assignments are still under discussion. For example, the weak band at 1445 cm^{-1} which is formed in the beginning of the reaction and persists until the end, combined with a slight intensity increase and a red shift ($1445 \rightarrow 1438\text{ cm}^{-1}$) with reaction time, is not characteristic for “ordinary” PANI-ES [35] and may arise from chain branching (*e.g.* **B** and **D** dimers, **H** trimer in **Scheme 2**) and/or intramolecular cyclization (*e.g.* **F** dimer, **K** trimer in **Scheme 2**). Generally, it is attributable to ring C=C stretching vibrations [32], possibly in short chains/short branches, or in substituted phenazine- and *N*-phenylphenazine-type oligomers. Indeed, the Raman spectra of phenosafranine and its oligomers have a band at about 1450 cm^{-1} that was assigned to ring stretching vibrations [29] and to substituted phenazine-type units formed by intramolecular oxidative cyclization processes, and pure phenazine has a weak Raman band at $\approx 1458\text{ cm}^{-1}$ [36]. This indicates, as already discussed in the previous section (UV/vis/NIR data), a relatively high abundance of phenazine-like structural units in the products obtained (**Scheme 2**). Another band which may be attributable to phenazine-type units can be seen at $1412\text{--}1416\text{ cm}^{-1}$, whose intensity decreases with increasing reaction time. The shoulders/ peaks at about 1570 cm^{-1} and 1550 cm^{-1} can also be attributed to phenazine-, phenoxazine-, or safranine type segments [33,34,36–38]. Finally, the band present at $\approx 1382\text{ cm}^{-1}$ already 1 min after starting the reaction decreases during the reaction until it disappears after 1 day (**Fig. S2**). The origin of this band is open for discussion. Possible assignments are $\nu(\text{C}-\text{N}^+)$ vibrations in *N*-phenylphenazine units, and/or $\nu(\text{C}-\text{N}^+)_p$ vibrations in localized polaron sites [33].

Overall, the *in situ* Raman spectra of the reaction mixtures of PADPA with HRPC/H₂O₂ at *pH* = 4.3 in the presence of AOT vesicles are similar to the *in situ* Raman spectra of the reaction mixtures of PADPA with TvL/O₂ at *pH* = 3.5 in the presence of the same type of vesicles [8]. However, one of the most important differences is seen regarding the band at $\approx 1440\text{ cm}^{-1}$, which is relatively strong for the system with HRPC/H₂O₂, while it

is relatively weak for the system with TvL/O₂ (positioned at $\approx 1450\text{ cm}^{-1}$) [8]. Based on this difference and on the absence of a 600 nm band in the vis spectrum of the products obtained with TvL/O₂, we conclude that the absorption at $\approx 600\text{ nm}$ in the vis spectrum (**Fig. 2**) is most probably related to short chains containing phenazine-type units.

Based on the comparison of the *in situ* Raman spectra of the oligo(PADPA) products formed in advanced stages of the reactions with the vesicles (*e.g.* 3 days) using HRPC/H₂O₂ (**Figure S2**) and TvL/O₂ [8], it can be deduced that the mass fraction of products with structural “defects” (such as *N*-phenylphenazine and/or phenazine-like structures, branches) in relation to the mass fraction of PANI-ES-like products is higher in the case of the reaction with HRPC/H₂O₂ than for the reaction with TvL/O₂ [8]. The influence of a different starting *pH* value (4.3 vs. 3.5 in cases of HRPC/H₂O₂ and TvL/O₂, respectively) on the mechanism of the PADPA oligomerization and the subsequent distribution of the different products is possible, similarly to the chemical polymerization of aniline [42,43] where a lower *pH* acts in favor of the formation of more regular, *para*-coupled linear chains.

3.1.5. In situ Raman spectroscopy measurements of the reaction in the absence of vesicles

The fluorescence background was very strong in the case of the reaction performed in the absence of AOT vesicles. This may be the reason for more noisy Raman spectra of this system (template-free system, **Fig. 6** and **Fig. S3**) compared with the spectra of the reaction mixture with vesicles (**Fig. 5** and **Fig. S2**).

Bands typical for PANI-emeraldine base (EB) and PANI-ES in the spectra of intermediates and products formed in the absence of vesicles:

One of the most important differences between the Raman spectra recorded during the reaction of PADPA with HRPC/H₂O₂ in the presence and absence of AOT vesicles is that

without vesicles, the $\nu(\text{C}\sim\text{N}^{++})_{\text{P}}$ bands are either absent or weak. For longer reaction times (see **Fig. S3**), the $\nu(\text{C}\sim\text{N}^{++})_{\text{P}}$ band at about 1340 cm^{-1} [35,39,40] is somewhat stronger but its relative intensity is still much lower than that of the $\nu(\text{C}\sim\text{N}^{++})_{\text{P}}$ band in the corresponding spectra for the reaction with AOT vesicles (see **Fig. S2**). This suggests that the conductivity of the reaction products obtained without vesicles must be low. Bands which can nevertheless be attributed to standard PANI structures can be found, see **Table 1**. These include functional groups such as SQ, Q and B structures. Furthermore, in the spectra recorded at longer reaction times ($t = 1, 3$ and 19 days, **Fig. S3**) a new band appears at about $1485\text{--}1495\text{ cm}^{-1}$ which can be attributed to the C=N stretching vibrations in quinonodimine (QD) units, $\nu(\text{C}=\text{N})_{\text{QD}}$. The presence of such a band is *not* characteristic of PANI-ES but is instead typical of non-conducting forms of PANI (*e.g.* the emeraldine base, PANI-EB) [33].

Bands atypical for PANI-EB and PANI-ES in the spectra of intermediates and products formed in the absence of vesicles:

Again, as was found in the reaction with vesicles, bands which are atypical of PANI are also formed in the absence of vesicles. Two bands which indicate the presence of phenazine, *N*-phenylphenazine (**Scheme 2**), phenoxazine or safranine structures were found at $1644\text{--}1630\text{ cm}^{-1}$ and at 1564 cm^{-1} , with a red shift observed at later times ($1564 \rightarrow 1546\text{ cm}^{-1}$). The shoulder at $\approx 1677\text{ cm}^{-1}$ observed at times before 90 min and the peak observed at $\approx 1690\text{ cm}^{-1}$ at $t = 90$ min and 140 min (**Fig. 6**) and later (**Fig. S3**) correspond to $\nu(\text{C}=\text{O})$ vibrations [32] and indicate the likely hydrolysis of iminoquinonoid (C=N) groups to carbonyl groups (C=O) at oligo(PADPA) chain ends [34]. This hydrolysis does not seem to occur to a large extent when AOT vesicles are present in the system (see also 3.1.7).

The *in situ* recorded Raman spectra of the reaction of PADPA with HRP/C/H₂O₂ at $\text{pH} = 4.3$ without vesicles are significantly different from the *in situ* recorded Raman spectra of

the reaction of PADPA with TvL/O₂ at $pH = 3.5$ *without* vesicles [8]. Therefore, the reaction mechanisms with the two catalyst/oxidant systems must be different. One of the most important differences is that in the early period, $\nu(C-N^{++})_P$ bands are clearly seen in the spectra for the template-free TvL/O₂ system [8], while for the template-free HRPC/H₂O₂ system these bands are not present at all during the same time period (**Fig. 6**). This is an indication that a C–N type of coupling between PADPA molecules and the formation of cation radical structures $(C-N^{++})_P$ does not take place during the early stage in the template-free HRPC/H₂O₂ system. This is in full agreement with the EPR spectra (**Fig. S1**) and the HPLC analysis (see below) of the reaction without vesicles. Furthermore, the Raman spectra of oligo(PADPA) obtained with HRPC/H₂O₂ in the template-free system support the formation of oxygen-containing products, as was found previously for the reaction of PADPA with TvL/O₂ without vesicles [8,22].

3.1.6. Changes in the HRPC activity during the reaction of PADPA with HRPC/H₂O₂

The peroxidase activity of HRPC in the reaction suspension was analyzed with ABTS²⁻ / H₂O₂ during the course of the reaction. The results are shown in **Fig. 7**. They indicate that the activity of HRPC rapidly decreases to about 70 % of the initial value after a reaction time of only 1 min, both for the reaction mixture with and without vesicles. After 13 min, there is almost no HRPC activity left in the reaction with vesicles, while in the reaction without vesicles, HRPC still retains 26 % of its initial activity. After at least 4 h, the enzyme is completely inactive in both systems. This suggests that the observed spectroscopic changes for the reaction with vesicles after 10–40 min, see **Fig. 2** (*e.g.* increase in $A_{1060\text{ nm}}$) and **Fig. 4** (increase in concentration of unpaired electrons) are *not* due to a direct involvement of active HRPC. This is similar to what was found in the case of the AOT vesicle assisted oxidation and polymerization of aniline with HRPC/H₂O₂, see [3].

3.1.7. HPLC – DAD and HPLC – MS measurements

High performance liquid chromatography (HPLC) coupled with a diode array detector (DAD) or a mass spectrometer (MS) complements the Raman spectroscopy measurements. In the manner in which the samples were prepared for HPLC (extraction, deprotonation, reduction [22]), information on the protonation and redox states of the products is lost. However, valuable information is obtained on the number of products formed, the individual UV/vis spectra, the polarity, the mass and thus molecular formula, and, with selective deuteration [22], the constitution of the deprotonated and reduced products. Thus, chromatograms of the reaction products extracted at selected times from the reaction with and without vesicles were recorded at 310 nm, using a reverse phase column with a water-acetonitrile gradient [22]. The molecular masses of the detected peaks were determined with the MS. The same was then done for the products formed from selectively deuterated PADPA, where the entire second ring of PADPA was deuterated (D5-PADPA, [22]). By determining the exact number of deuterium atoms in the various products by using the MS as a detector, the localization of the bond formation site of two or more PADPA molecules with each other for the formation of a certain product, *i.e.* coupling modes, can be determined, *e.g.* whether there is a coupling in *para*- or *ortho*- position related to the NH₂ group. This gives information on different reaction routes. For more detailed information on the sample preparation, see Luginbühl *et al.* [22].

Products formed with AOT vesicles:

The time-dependent chromatograms of the reaction with vesicles are shown in **Fig. 8a** up to a reaction time of 24 h. For the sake of clarity, the chromatogram after 2 h is also shown in **Fig. 8c** (top). Immediately after starting the reaction (reaction time (rt) of ~5 s), not only the peak originating from PADPA is visible at rt ~20 min, but also several other peaks

originating from intermediate products. During the next minutes, the PADPA peak steadily decreases but is still visible after 120 min (peak 1 in **Fig. 8c**), or even after 24 h. The peak with a *rt* of 21 min (peak 2 in **Fig. 8c**) was determined to be the hydrolyzed form of PADPA, which is formed by replacing the terminal amino group with a hydroxyl group. With vesicles, its content seems to slightly decrease over 2 h. The peak at *rt* = 34 min (peak 4 in **Fig. 8c**) stems from the linear, N_{prim}-C₁₀ coupled PADPA dimer (PADPA)₂ (**Scheme 2**) which was determined to be the main product of the reaction of PADPA with TvL/O₂ with vesicles [22]. It is most likely that this species is primarily responsible for the typical PANI-ES bands in the Raman spectra (see **Table 1**). The intensity of the peak at *rt* = 34 min after 1 h is about one quarter of that of the TvL/O₂ system after the same reaction time. In the reaction with HRPC/H₂O₂, the peak from (PADPA)₂ appears suddenly after a reaction time of ~5 s and increases in intensity until 2 h (peak 4 in **Fig. 8c**). Peak 3 in **Fig. 8c** is most likely an *aniline* trimer, and peak 5 a PADPA trimer, both of unknown constitution. Finally, there is a cluster of peaks with a *rt* between 52 and 60 min (the main peaks are labeled with 6, 7, and 8 in **Fig. 8c**). A high *rt* indicates that these molecules are quite apolar (or long!) and thus are structurally different from (PADPA)₂. In addition, their UV/vis spectra are also fundamentally different than the UV/vis spectrum of (PADPA)₂, see **Fig. S4**. The spectra of the peaks eluting with high *rt* have all a maximum at ≈ 500 nm. MS analysis shows that all these peaks at higher *rt* stem from PADPA oligomers with three to five PADPA units ((PADPA)₃₋₅) and their corresponding hydrolysis products. For example, the peak with *rt* = 56 min (peak 7 in **Fig. 8c**) stems from a PADPA trimer. This oligomer, and the others, are not typical PANI chains, and must be formed by two or more bond formation steps *via* coupling in *ortho*-position, based on the HPLC-MS measurement with D5-PADPA, see also [22]. Cyclization reactions after C–N coupling in *ortho*-position to NH₂ group are very likely to occur, leading to the formation of colored, phenazine-type oligomers. Thus, the HPLC-DAD and HPLC-MS

measurements complement the UV/vis and Raman spectroscopy analysis, which indicate that a significant number of phenazine products are formed, with a higher abundance than in the reaction of PADPA with TvL/O₂ [8,20]. In addition, in the reaction of PADPA with HRPC/H₂O₂ in the presence of vesicles, all dominant products were found to have undergone to some extent hydrolysis – PADPA, (PADPA)₃₋₅ – except (PADPA)₂. This (a) demonstrates the importance of intact, non-hydrolyzed (PADPA)₂ for obtaining the expected PANI-ES signal response in EPR and UV/vis spectroscopy, and (b) is another big difference between this reaction and the reaction of PADPA with TvL/O₂ in the presence of AOT vesicles, where *no* hydrolyzed products were found at all [22].

Products formed in the absence of vesicles analyzed with HPLC

The HPLC chromatograms of the reaction without vesicles are shown in **Fig. 8b** for reaction times up to 24 h. For a reaction time of 2 h, see also **Fig. 8c** (bottom). The UV/vis spectra of the two main chromatographic peaks (a and b in **Fig. 8c**) are given in **Fig. S5**. As discussed above for the reaction with vesicles, at reaction time of ~5 s, not only PADPA (at *rt* = 20 min), but also a small amount of hydrolyzed PADPA is visible at a *rt* = 21 min. This latter peak develops and steadily increases during the reaction. For the first 60 min of the reaction, no other products are formed. This roughly coincides with the Raman spectroscopy measurements, where no polaron signals are seen for the first phase of the reaction (**Fig. 6**). Later on, after ~60 min, other products are nevertheless formed. They are different from the ones obtained with vesicles, and are formed to a much lower extent. Weak evidence for the formation of (PADPA)₃₋₅, hydrolyzed (PADPA)₃₋₅, and both non-hydrolyzed and hydrolyzed (PADPA)₂ was found. Analysis of the reaction mixtures after 24 h showed that product transformations still occurred in the template-free system if the reaction mixture is kept for more than 1 h (**Fig. S6a**, bottom). The most intense peak after 24 h is at *rt* = 34 min (peak **c** in

Fig. S6a bottom and **Fig S6b**). It originates from hydrolyzed (PADPA)₂, most likely formed via a coupling reaction of PADPA and hydrolyzed PADPA. In contrast, in the presence of vesicles there was no further change in the product distribution obtained after a reaction time of 2 h (see **Fig. S6a**, top).

To summarize, these measurements show that with AOT vesicles, the yield of hydrolyzed PADPA is minimized, the hydrolysis of (PADPA)₂ is completely suppressed, and the formation of (PADPA)₂ as PANI-ES is favored. Thus, the HPLC-MS measurements show that the vesicles (i) prevent hydrolysis, (ii) increase the formation of (PADPA)₂, and (iii) provide the optimal environment so that the protonation and oxidation state of (PADPA)₂ resembles that of PANI-ES (N_{prim}-C₁₀ coupled PANI-ES, **Scheme 2**).

3.2. Cyclic voltammetry measurements

The reaction mixture can be deposited on a glassy carbon electrode for cyclic voltammetry measurements *without* preceding isolation and purification of the products [8]. In this way, a complex pH-dependent electrochemistry of oligo(PADPA) was observed (**Fig. 9**), as previously with the TvL/O₂ system [8]. The most important observation is that the redox activity of the AOT-containing products is preserved at increased pH values up to pH = 6.00, which points to a beneficial role of AOT vesicles combined with enzymatic oligomerization, as PANI typically loses its electro-activity when the pH is above 5 [44]. Despite the complexity of the current response and the well-known effect of anions [45,46], which make it difficult to provide a detailed mechanism of the electrochemical reactions of oligo(PADPA), the electrochemical behavior reveals the crucial role of the catalyst/oxidant and AOT vesicles on the obtained products.

At pH = 1.14, the cyclic voltammogram of oligo(PADPA) produced in the presence of AOT vesicles (**Fig. 9A**, curve 1) is similar to that of PANI [45]. Two anodic peaks are

observed at 0.3 and 0.56 V *vs.* SCE, while the corresponding cathodic peaks are located 0.17 and 0.43 V *vs.* SCE. As well accepted in the literature, the redox couple located at lower potentials is ascribed to the interconversion between the leucoemeraldine and the ES form of PANI-like segments, while the second redox couple found at higher anodic potentials is due to the redox transformations of the ES to the pernigraniline form [45]. Although the cyclic voltammograms of oligo(PADPA) products formed with HRPC/H₂O₂ in the presence of vesicles evolve in a similar way with increasing pH as the cyclic voltammograms of the products obtained with the TvL/O₂ system do [8] (**Fig. 9, A-C**), in the HCl containing solutions (pH=1.14 and 3.04) the current response associated with emeraldine ↔ pernigraniline transformations is more pronounced than in the case of the TvL/O₂ system. This supports the general findings of the spectroscopic measurements, suggesting that the catalyst/oxidant (HRPC/H₂O₂ *vs.* TvL/O₂) has a clear influence on the products obtained. At the upper pH limit investigated here (pH=6.00), the successive anodic and cathodic processes almost completely merge, resulting in overlapped current peaks associated with oxidation and reduction of oligo(PADPA) (**Fig. 9 C**).

The electrochemical behavior of the oligo(PADPA) products obtained with and without vesicles is quite different. Similarly to oligo(PADPA) produced with TvL/O₂ [8], the products formed without vesicles were found to be redox active only under very acidic conditions, pH = 1.14 (**Fig. 9A**, curve 2), which is possibly due to the much lower conductivity of the products in comparison to their counterparts obtained in the presence of vesicles. It is clear that the presence of AOT vesicles is essential for the preservation of the redox activity of the oligo(PADPA) products at high *pH* values, which is important for numerous applications.

3.3. Insight into the chemical structure-controlling (template) effect of AOT vesicles

The results of the present and our recent investigations [7,8,22,23], by UV/vis/NIR, EPR, Raman, HPLC and CV, of the oligo(PADPA) products synthesized enzymatically with and without vesicles confirm that AOT vesicles act as a *chemical structure-controlling agent (template)* during the oxidation/oligomerization of PADPA with both the HRPC/H₂O₂ and the TvL/O₂ enzyme/oxidant systems, enabling a high abundance of products in *polaronic ES-like form*, mainly (PADPA)₂. The vesicles encourage the coupling of two PADPA molecules towards (PADPA)₂ and prevent the formation of hydrolyzed (PADPA)₂; the vesicles promote one reaction pathway and prevent another one [22]. Based on the literature [28], (PADPA)₂ and higher oligomers of PADPA in ES-like form obtained in the presence of vesicles must exist predominantly in an *expanded coil conformation*, as strongly indicated by the absorption band at ~1000 nm in the UV/vis/NIR spectrum (**Fig. 1**). On the other hand, in the analogous systems *without vesicles*, the results showed that the structures of the oligomeric products *do not contain delocalized polarons* or their amount is negligible. The templating effect of AOT is partially a *conformation-controlling and electronic structure-controlling effect*. These effects are due to the negative surface charge of AOT vesicles, due to R-SO₃⁻ groups. Thus, the AOT surface electrostatically attracts the positively charged radical cations (–NH^{•+}). This may stabilize the *expanded coil conformation* of (PADPA)₂ and higher oligomers in ES-like delocalized polaron form (polaron lattice) and provide PADPA and its intermediate with a microenvironment (and a low water activity), inhibiting hydrolysis [22]. In addition, there is a high local concentration of PADPA on the vesicles' surface, which encourages coupling towards (PADPA)₂ and (PADPA)₃₋₅. AOT anions act as dopant anions (counter-ions) for the positively charged oligomeric backbone. Besides attractive electrostatic ion-ion interactions

between a positively charged (PADPA)₂ molecule in its ES form and the negatively charged outer surface of an AOT vesicle (**Scheme 3a**), hydrogen bonds of O ...HN type between the O atom in the carbonyl or sulfonate groups of AOT and the H atom from the NH⁺ groups of PADPA oligomers are additional possible attractive interactions (**Scheme 3b**). The H-bonding interaction could also be three-centered (bifurcated), where the H atom from an NH⁺ group simultaneously interacts with the O atom from the carbonyl group and an O atom from the sulfonate group, forming a six-membered ring (**Scheme 3b**) [47]. Once (PADPA)₂ is formed, it is likely that the average distance between two adjacent AOT molecule heads at the vesicle surface corresponds well to the distance between two adjacent positively charged nitrogen atoms in the delocalized ES form of (PADPA)₂ (or higher ES-like oligomers), as illustrated in **Scheme 3**. Such appropriate structural geometry may act in favour of internal rearrangement of charged dication structures (bipolarons) to delocalized polaron structures. This hypothesis remains to be proven.

4. Conclusions

There are the following main conclusions which can be drawn from the results obtained in this basic research study.

(i) PADPA can be oxidized with small amounts of HRPC and with H₂O₂ as oxidant to yield under optimal reaction conditions in the presence of AOT vesicles oligo(PADPA) products which resemble PANI-ES in its polaron state (see **Scheme 1**). The main product is (PADPA)₂. For a conversion of 1.0 g PADPA, ≈7 mg HRPC is required, as calculated by taking into account M(PADPA) = 184.2 g/mol and M(HRPC) = 44000 g/mol [48]. This is comparable to the situation with TvL/O₂. In both cases, the oxidation of PADPA is much more efficient than the oxidation of aniline [3, 5], see Introduction.

- (ii) The oligo(PADPA) products obtained with HRPC/H₂O₂ in the presence of AOT vesicles undoubtedly contain detectable amounts of phenazine-type products (UV/Vis/NIR absorption at ≈ 600 nm and Raman measurements) and possibly phenoxazines; both in a higher abundance than in the case of oligo(PADPA) obtained with TvL/O₂ with vesicles [8].
- (iii) PADPA and the longer PADPA oligomer products, (PADPA)₃₋₅, obtained with HRPC/H₂O₂ in the presence of vesicles contain oxygen atoms (HPLC-MS analysis of the extracted, deprotonated and reduced products) as a result of hydrolytic reactions, occurring in both cases already at the very early stage of the reaction. This is different from oligo(PADPA) obtained with TvL/O₂, where the presence of AOT vesicles completely suppress all hydrolysis reactions [22]. Whether these differences are due to the different *pH* values used for the reactions or whether they are due to the differences in the enzyme/oxidant system remains to be clarified.
- (iv) The oligo(PADPA)-AOT vesicle suspension obtained with HRPC/H₂O₂ can be used for coating glassy carbon electrodes. The resulting film not only has a redox activity at $\text{pH} \approx 1.0$ or ≈ 3.0 (typical for PANI-ES [44]), but also at $\text{pH} \approx 6.0$ (CV measurements), which is potentially useful for numerous applications such as in the field of electrochemical biosensors. The same was also observed for the oligo(PADPA)-AOT vesicle suspension obtained with TvL/O₂ [8]. The product obtained without vesicles is redox active only under very acidic conditions ($\text{pH} \approx 1.1$).

Independent of whether PADPA is oxidized/oligomerized with HRPC/H₂O₂ (this work) or with TvL/O₂ [7,8,22], the AOT vesicles have a positive effect as “templates” on the outcome of the reaction (formation of PANI-ES-like compounds as main products, decreased or absent hydrolytic side reactions). It is, however, also clear that oligo(PADPA) is a mixture of compounds and not an individual molecule. Obtaining a mixture of different compounds

instead of one single product may not necessarily be a disadvantage in terms of properties, it may even be an advantage (especially concerning the electrochemical behavior). This is what needs to be explored further. Studies on the application of the as-obtained oligo(PADPA)-AOT vesicle suspension containing PANI-ES-like products are ongoing. Due to the high colloidal stability, which is one criteria during the reaction optimization (no product precipitation, see Introduction), we have already demonstrated that the as-obtained reaction suspensions can not only be used for coating electrodes, but also as ink in a commercial inkjet printer [5]. This makes handling the reaction products much easier, similarly to most of the other template- assisted enzymatic polymerization reactions [11-20,24].

Acknowledgements. The financial support by the Swiss National Science Foundation (SCOPES Joint Research Project IZ73Z0_152457 and 200020_150254) and Ministry of Education, Science and Technological Development of the Republic of Serbia (project OI172043) is highly appreciated.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at ...

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2.0 mM AOT
 0.1 M NaH_2PO_4 , $\text{pH} = 4.3$
 $T \approx 25^\circ\text{C}$

1.0 mM PADPA
 30 nM HRPC, 1.0 mM H_2O_2

Scheme 1. Schematic representation of the anionic vesicle system investigated with indication of the optimal reaction conditions. Unilamellar AOT vesicles with an average diameter of $\approx 80\text{-}100\text{ nm}$ were used as templates at $\text{pH} = 4.3$ ($0.1\text{ M NaH}_2\text{PO}_4$) and $T \approx 25^\circ\text{C}$. The monomer PADPA binds to the negatively charged vesicle surface and is oxidized by HRPC – which itself is activated (oxidized) by H_2O_2 – to the PADPA radical cation ($\text{PADPA}^{\bullet+}$). Subsequent coupling reactions lead to the formation of oligo(PADPA), which is a mixture of oligomeric products.

Scheme 2. Possible PADPA dimers and trimers obtained from PADPA with HRPC/H₂O₂ in the presence of AOT vesicles: **A)** N_{prim}–C₁₀ coupled dimer in the reduced form, **B)** N_{prim}–C₃ coupled dimer in the reduced form, **C)** N_{prim}–C₁₀ coupled dimer in the half-oxidized PANI-ES-bipolaron-like form, **D)** N_{prim}–C₃ coupled dimer in the half-oxidized bipolaron form, **E)** N_{prim}–C₁₀ coupled dimer in the half-oxidized PANI-ES-polaron-like form, **F)** N_{prim}–C₃ coupled dimer in the phenazine form, **G)** N_{prim(A)}–C_{10(PADPA)} coupled linear trimer in the reduced form, **H)** N_{prim(B)}–C_{10(PADPA)} coupled branched trimer in the reduced form, **I)** N_{prim(A)}–C_{10(PADPA)} coupled trimer in the partially-oxidized bipolaron-like form, **J)** N_{prim(A)}–C_{10(PADPA)} coupled trimer in the half-oxidized PANI-ES polaron-like form, **K)** N_{prim(B)}–C_{10(PADPA)} coupled trimer in the *N*-phenylphenazine form.

Figure 1. UV/vis/NIR spectra for the oxidation of PADPA (1.0 mM) with HRPc (30 nM) and H₂O₂ (1.0 mM) in aqueous solution at pH = 4.3 (0.1 M H₂PO₄⁻) and $T \approx 25$ °C, $t = 24$ h, either in the presence of AOT vesicles at 2.0 mM AOT (spectrum 1, solid line) or without vesicles (spectrum 2, dotted line, template-free system); $V_r = 10$ mL, see Experimental Part. Spectrum 2 is the spectrum which was measured after dispersing the precipitates that formed in the template-free system. The wavelength values given in the graph refer to the positions of the band maxima of spectrum 1.

Figure 2. Oxidation of PADPA (1.0 mM) with HRPc (30 nM) and H_2O_2 (1.0 mM) in aqueous solution at pH = 4.3 (0.1 M H_2PO_4^-) in the presence of AOT vesicles at 2.0 mM AOT and $T \approx 25^\circ\text{C}$. Time dependent changes of the UV/vis/NIR absorption spectrum of the reaction mixture during the course of the reaction. (a) Spectra recorded between 10 s (green thick line) and 2 min (black thick line) after starting the reaction; (b) spectra recorded before

starting the reaction (1) and between 2 min (2, red line) and 60 min (5, green line); (c) spectra recorded between 60 min (5, green line) and 4 h (9, black line); (d) changes in A_{1060} , A_{850} , A_{600} , A_{415} , and A_{300} during the reaction. The wavelength given in (a) – (c) refer to the positions of the band maxima. The arrows in (a) – (c) indicate the directions of the spectral changes with time. Please note that the spectra recorded in (a) were carried out with a diode array spectrophotometer with a wavelength range between 190 and 1000 nm; for (b) and (c), $V_r = 10$ mL, see Experimental Part.

Figure 3. Oxidation of PADPA (1.0 mM) with HRPC (30 nM) and H_2O_2 (1.0 mM) in aqueous solution at $\text{pH} = 4.3$ ($0.1 \text{ M H}_2\text{PO}_4^-$) and $T \approx 25^\circ\text{C}$ *without* any vesicles (template-free system). Time dependent changes of the UV/vis absorption spectrum of the reaction mixture at the beginning of the reaction. The spectra were recorded with a diode array spectrophotometer between 20 s (black thick line) and 2 min s (red thick line) after starting the reaction.

Figure 4. Oxidation of PADPA (1.0 mM) with HRPc (30 nM) and H_2O_2 (1.0 mM) in aqueous solution at $\text{pH} = 4.3$ ($0.1 \text{ M H}_2\text{PO}_4^-$) in the presence of AOT vesicles at 2.0 mM AOT and $T \approx 25^\circ\text{C}$; $V_r = 10 \text{ mL}$, see Experimental Part. Time dependent changes of the EPR spectrum of the reaction mixture during the course of the reaction. (a) Spectra recorded at 1, 2, 4, and 10 min after starting the reaction; (b) spectra recorded at 20, 60, 90, and 120 min after starting the reaction; (c) changes in the integral of the EPR signal between 3450 and 3510 G during the reaction. For (a) and (b), dA/dB is the derivative of the absorption.

Band [cm ⁻¹]	Functional group <i>B = benzenoid</i> <i>Q = quinonoid</i> <i>SQ = semiquinonoid</i> <i>QD = quinonediimine</i>	typical for PANI - ES <i>y = yes</i> <i>n = no</i>	vibration type ^x	Reaction <i>AOT = with vesicles</i> <i>TF = without vesicles</i>	Comments
1179 – 1192	SQ B	y y	$\delta(\text{C}\sim\text{H})_{\text{SQ}}$ $\delta(\text{C}\sim\text{H})_{\text{B}}$	AOT/TF	
1222 – 1225 1263 – 1266	B	y	$\nu(\text{C}\sim\text{N})_{\text{B}}$	AOT AOT/TF	
1315 – 1320	polarons	y	$\nu(\text{C}\sim\text{N}^{+})_{\text{p}}$	AOT	delocalized
1346 – 1355	polarons	y	$\nu(\text{C}\sim\text{N}^{+})_{\text{p}}$	AOT/TF	localized
1382	<i>N</i> -phenylphenazine or localized polaron	n	$\nu(\text{C}\sim\text{N})$ or $\nu(\text{C}\sim\text{N}^{+})$	AOT	unclear
1412 – 1416	phenazine/safranine	n	$\nu(\text{ring})$	AOT	
1438 – 1444	branching or intramolecular cyclization/ substituted phenazine oligomers	n	$\nu(\text{C}=\text{C})$	AOT	unclear
1485 – 1495	QD	n	$\nu(\text{C}=\text{N})_{\text{QD}}$	TF	typical for PANI-EB
1511 – 1515	SQ	y	$\delta(\text{N}\sim\text{H})_{\text{SQ}}$	AOT/TF	
1546 – 1564	phenazine/safranine/phenoxazine	n	?	TF	unclear
1592 – 1597	SQ Q	y y	$\nu(\text{C}\sim\text{C})_{\text{SQ}}$ $\nu(\text{C}=\text{C})_{\text{Q}}$	AOT/TF	
1607 – 1616	B		$\nu(\text{C}\sim\text{C})_{\text{B}}$	AOT	new type of aromatic ring
1624	B	y	$\nu(\text{C}\sim\text{C})_{\text{B}}$	AOT	
1630 – 1644	phenazine, <i>N</i> -phenylphenazine, phenoxazine convoluted with B	n	$\nu(\text{C}\sim\text{C})$	TF	
1697	carbonyl	n	$\nu(\text{C}=\text{O})$	TF	hydrolysis

Table 1. Overview of the Raman bands for the reaction oxidation of PADPA with HRPC/H₂O₂, including their assignments to functional groups and vibration types [32–41].

^xVibration type: ν = stretching vibration, δ = bending vibration. In parenthesis, the two atoms involved in the vibration are given.

Figure 6. *In situ* Raman spectra of the reaction mixture recorded at various times during the oxidation reaction of PADPA (1.0 mM) with HRPC (30 nM) and H₂O₂ (1.0 mM) in aqueous solution at pH = 4.3 (0.1 M H₂PO₄⁻) and *T* ≈ 25 °C *without* any vesicles (template-free system), V_r = 10 mL, see Experimental Part. The reaction times at which the spectrum was measured were 1, 5, 12, 20, 30, 40, 52, 60, 90, and 140 min (from bottom to top).

Figure 7. Changes in the activity of HRP during the oxidation/oligomerization of PADPA (1.0 mM) with HRP (30 nM) and H_2O_2 (1.0 mM) in aqueous solution at $\text{pH} = 4.3$ ($0.1 \text{ M } \text{H}_2\text{PO}_4^-$) and $T \approx 25^\circ\text{C}$, either in the presence of AOT vesicles at 2.0 mM AOT (filled circle) or without vesicles (empty square, template-free system). $V_r = 10 \text{ mL}$; the activity was measured at room temperature with $0.25 \text{ mM } \text{ABTS}^{2-}$ as a substrate and $50 \mu\text{M } \text{H}_2\text{O}_2$ as an oxidant at $\text{pH} = 6.0$, see Experimental Part for details. The values given are relative values which are related to the activity determined immediately before starting the reaction ($t = 0 \text{ min}$). The two dotted lines mark $t = 1$ and 2 hours , respectively.

Figure 8. HPLC-DAD results of the reaction with vesicles (a) and without vesicles (b). Reaction mixtures: 1.0 mM PADPA, 30 nM HRPc, 1.0 mM H_2O_2 , pH = 4.3 (0.1 M $H_2PO_4^-$), $T \approx 25^\circ C$, either in the presence of AOT vesicles at 2.0 mM AOT (a) or without vesicles (b);

$V_r = 10$ mL, see Experimental Part. Samples were withdrawn from the reaction mixtures, deprotonated with ammonia, extracted into methyl-*t*-butylether (MTBE) and then reduced with hydrazine (N_2H_4); afterwards, the MTBE solutions containing a mixture of reduced products were applied onto a reversed phase C18 column, see Luginbühl *et al.* (2016) for the experimental details [22]. In (c), a comparison is made of the chromatograms as obtained after a reaction time of 2 h with AOT vesicles (top) and without vesicles (bottom). The absorption spectra of the main peaks (1-8 and a,b) are given in the Supporting Information, **Fig. S4** and **Fig. S5**.

Figure 9. Cyclic voltammograms of GC electrode modified by direct drop-casting the final reaction suspension with AOT vesicles (red circles, curve 1) recorded at different pH: A – 1.14, B – 3.04, C – 6.00. Voltammograms were recorded in quiescent N_2 -purged solutions at a common polarization rate of 20 mV s^{-1} . At pH = 1.14 oligomerization product obtained in the template-free system was also found to be redox active (blue circles, curve 2).

Scheme 3. Schematic illustration of the suggested a) attractive electrostatic ion-ion interaction between a positively charged $(\text{PADPA})_2$ molecule in its separated polaron state (ES form) and the negatively charged outer surface of an AOT vesicle, b) hydrogen bonding interactions between $(\text{PADPA})_2$ molecule in its ES form and AOT at the vesicle surface; the dashed lines indicate possible H-bonding, whereby the H-bond to the sulfonate O-atom is probably favored; the H-bonding could also be three-centered (bifurcated), including simultaneously H atom from NH^+ group, O atom from carbonyl group, and O atom from sulfonate group, and forming a six-membered ring.