RESEARCH ARTICLE

Green synthesis of conductive polyaniline by *Trametes versicolor* laccase using a DNA template

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The green synthesis of highly conductive polyaniline by using two biological macromolecules, i.e. laccase as biocatalyst, and DNA as template/dopant, was achieved in this work. *Trametes versicolor* laccase B (TvB) was found effective in oxidizing both aniline and its less toxic/mutagenic dimer N-phenyl-p-phenylenediamine (DANI) to conductive polyaniline. Reaction conditions for synthesis of conductive polyanilines were set-up, and structural and electrochemical properties of the two polymers were extensively investigated. When the less toxic aniline dimer was used as substrate, the polymerization reaction was faster and gave less-branched polymer. DNA was proven to work as hard template for both enzymatically synthesized polymers, conferring them a semi-ordered morphology. Moreover, DNA also acts as dopant leading to polymers with extraordinary conductive properties (~6 S/cm). It can be envisaged that polymer properties are magnified by the concomitant action of DNA as template and dopant. Herein, the developed combination of laccase and DNA represents a breakthrough in the green synthesis of conductive materials.

KEYWORDS

biopolymer synthesis, electrochemical impedance spectroscopy, laccase, morphological characterization, polyaniline

1 | INTRODUCTION

Polyaniline is an important intrinsically conducting polymer with three different oxidation states. Among them, only emeraldine, the half-oxidized form was demonstrated to be conductive. The processability and conductivity of polyaniline can be modulated during the synthesis, by using different types of dopants and templates that help the linear head-to-tail coupling avoiding unwanted side-chain branching reactions [1]. There are many reports showing different ways

to produce conductive polyaniline, classically starting from aniline monomer under strong acidic conditions. Most of conventional processes of synthesis are not environmentally friendly due to the use of high concentration of strong acids and oxidants, and toxic solvents. More recently, methods to produce polyaniline starting from the less toxic aniline dimer (DANI) [2] have been developed [3,4]. Reaction parameters affect polymer morphology both at micro- and nanoscale levels. As a fact, a wide variety of structures have been described for polyaniline synthesized by different routes [5]. Due to its intrinsically conductivity and peculiar morphological flexibility, polyaniline has many potentialities for practical applications (batteries, energy-storage devices, anticorrosion protection, antistatic coating) and can contribute to the field of nano/macro science [1,6].

Abbreviations: ABTS, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; DANI, Dimer of aniline; EIS, Electrochemical impedance spectroscopy; FTIR, Fourier transformed infrared spectroscopy; SM, Stereomicroscope.

Biocatalytic processes, endowed with no induction period, simplified downstream processes and milder conditions, have been proposed as green alternatives to the classical chemical synthesis [7]. Both HRP and laccases have been successfully used as biocatalysts for polymerization of aniline and its dimer [7–9]. Laccases are multicopper oxidases able to oxidize many inorganic and organic substrates catalyzing the reduction of molecular oxygen to water [10]. Laccase-catalyzed synthesis have attracted great interests due to its eco-friendly features since laccases do not require addition of hydrogen peroxide using oxygen as oxidant. Biosynthesis of polyaniline has been performed in presence of various templates such as, surfactants, lignosulfonates, proteins, and nucleic acids [7–9,11–18]. The choice of the template is relevant in determining the appropriate chemical structure of conductive PANI, its morphology and, therefore, its final use. From a practical point of view, the achievement of water soluble or dispersible PANI is a very sought-after feature [19]. In this regard, anionic surfactants as well as micelles or vesicles-forming amphiphilic systems, used as templates, have been proven able to solubilize PANI. Among biological templates, DNA also allows the achievement of water dispersible polyaniline since the polymer can be wrapped around it [13–18,20]. The use of DNA templates is even more attractive since it offers a platform for the fabrication of PANI nanostructures reproducing the near-infinite number of rigid nanostructures potentially obtainable from DNA-self assembling [20–22]. The advent of DNA Origami technology, has further improved the flexibility of DNA assembly, allowing to create PANI nanopatterns with predesigned geometries with the origami templates [22]. Naturally existing DNA nanostructures, calf thymus DNA, have been used as templates for the synthesis of PANI nanowires in solutions or on the Si surface, using HRP as the catalyst [14]. On the other hand, PANI with controlled shapes on two-dimensional DNA origami templates has been achieved through the assembly of DNAzyme sequence within the origami [21].

The intrinsic biocompatibility of DNA templates adds a further feature to the conductive polymer, making it exploitable in the biomedical field. Moreover, due to the presence of negatively charged sites, DNA is of great interest also as dopant because its phosphate groups bind and align the aniline cations, boosting the head-to-tail coupling and promoting the synthesis of a conductive polymer [20].

The use of DNA template/dopant together with laccase as biocatalyst, has never been attempted so far, thus the set-up of this process could open the way to the synthesis of polymers endowed with different geometries/properties. Therefore, the purpose of the given work is to study the laccase catalyzed oxidation of aniline and its dimer exploring the effect of reaction conditions on polyaniline morphology and conductive properties.

PRACTICAL APPLICATION

Polyaniline is an important intrinsically conducting polymer finding applications in several fields (batteries, energy-storage devices, anticorrosion protection, antistatic coating) as well as in micro/nano science. The availability of an environment friendly process for its synthesis constitutes a further drive towards polyaniline application. The synthesis of highly conductive polyaniline by using two biological macromolecules, i.e laccase as biocatalyst, and DNA as template/dopant, was achieved in this work, representing a breakthrough in the green synthesis of conductive materials.

2 | MATERIALS AND METHODS

2.1 | Materials

The 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), the commercial PANI-ES ($M_w > 15\,000$ g/mol), aniline (ACS reagent 99.5% purity), acetone (spectrophotometric grade $\geq 99.5\%$), acetonitrile (99.9%), hydrochloric acid (reagent grade, 37%), DANI (N-phenyl-p-phenylenediamine 98%), DNA sodium salt from salmon testes, were purchased from Sigma–Aldrich. DANI was freshly prepared (1.5 mM) in buffer citrate pH 3.5 in an ultrasound bath. DNA sodium salt from salmon testes was freshly prepared 3 mM (1 mg/mL) in buffer citrate pH 3.5.

2.2 | Laccase purification

TvB laccase [23,24] was purified from *Trametes versicolor* NBRC 4937. *T. versicolor* was propagated on solid medium (PDY: 24 g/L potato dextrose, 5 g/L yeast extract). Liquid culture was carried out at 28°C inoculating 50 mL of PDY in 250 mL shaken flasks with 3 agar dishes with mycelium (11 mm diameter). 25 mL of a 5-day-old culture was homogenized and then used to inoculate in 1 L flasks containing 225 mL of PDY broth and 50 μ M CuSO_4 as inducer. Cultures were incubated at 28°C in a rotatory shaker at 150 rpm. After 48 h of growth, 1 mM vanillic acid was added to the culture as laccase inducer. After eight days of culture, the medium was collected and filtered through gauze. 1 mM of the serine protease inhibitor, PMSF, was added to the supernatant. Secreted proteins were precipitated from the filtered medium by addition of $(\text{NH}_4)_2\text{SO}_4$ up to 80% saturation and loaded on HiPrep™ Phenyl Sepharose High Performance 16/10 (GE Healthcare, Milan, Italy). Fractions corresponding to TvB were pooled, equilibrated in buffer 50 mM sodium phosphate (NaP) pH 6.5, and loaded onto a HiPrep™ MonoQ High Performance 16/10 column (GE Healthcare, Milan, Italy) with a

linear gradient from 0 M to 1 M NaCl, and fractions corresponding to TvB were pooled and desalted. The enzyme was purified to homogeneity according to SDS-electrophoresis.

POXC laccase from *Pleurotus ostreatus* was purified as described in Macellaro et al [25]; recombinant rPOXA1b was produced in *Pichia pastoris* and purified as reported in Pezzella et al. [26].

2.3 | Enzymatic activity assay

Laccase activity was assayed at room temperature by monitoring the oxidation of ABTS at 420 nm (ϵ_{420} 36×10^3 M⁻¹ cm⁻¹). The assay mixture contained 2×10^{-3} M ABTS in 0.1 M sodium citrate buffer, pH 3.0. Laccase activity towards 2,6-dimethoxyphenol (DMP) was assayed in a mixture containing 1×10^{-3} M DMP in McIlvaine's citrate phosphate buffer adjusted to pH 5.0. Oxidation of DMP was followed by an absorbance increase at 477 nm (ϵ_{477} 14×10^3 M⁻¹ cm⁻¹). One unit of activity is defined as the amount of laccase oxidizing 1 μ mol of substrate per min. Laccase activity towards aniline was monitored determining the Abs_{410nm} after 30 min of incubation of 300 mM aniline with 1 U/mL of enzyme at 28°C. Different enzymes were tested: the high-redox potential *T. versicolor* laccase B, TvB [23,27], *P. ostreatus*, POXC (E° 0.74, pH 5) [28], rPOXA1b (E° 0.65, pH 6) [26] and its improved variant 1H6C (E° 0.77, pH 6) [29].

2.4 | DNA- assisted enzymatic synthesis of polyaniline

Polymerization of aniline monomer was carried out in 0.1 M citrate buffer at pH 3.5 at 28°C for 24 h. The reaction was conducted at 150 rpm by using different amounts of TvB (1, 5, and 10 U/mL) in a final volume of 2 mL for analytical purposes, and scaled up to 20 mL for complete characterization of synthesized polymers. Different DNA/aniline ratios (2:1, 1:1, 1:2.5, 1:5) were tested in order to select the optimal reaction conditions. DNA concentration was fixed to 3 mM and aniline concentration was varied in the range of 1.5–15 mM to obtain the required ratios. Polymerization was followed by recording UV-Visible spectra (300–800 nm) at different times on a Jasco V-530 spectrophotometer. Absorption values at specific wavelengths typical for emeraldine salt (300, 400, and 730 nm), or indicative of formation of branched products (500 nm) were monitored during the time. Reactions without template were carried out by substituting the volume of DNA with the same volume of buffer solution. Polyaniline, PANI, was recovered through precipitation with Acetone (1:1 v/v) and dried in SavantTM SPD121P SpeedVacTM (Thermo Fisher Scientific) at 37°C for 30 min for further characterization [30].

Laccase-catalyzed polymerization of DANI was carried out in 0.1 M citrate buffer at pH 3.5 at room temperature to get

pDANI. The reaction was conducted at 150 rpm by using 1 U/mL of TvB in a final volume of 2 mL for analytical purposes, and scaled up to 20 mL for complete characterization of synthesized polymers. Different DNA/DANI ratios were tested in order to select the optimal reaction conditions. In the first trials, DANI concentration was set to 0.9 mM and DNA concentration to 1.2 mM (DNA/DANI ratio 1.3:1, as reported by Junker et al [9]). A higher DNA/DANI ratio in favor of DANI was also tested (1:1.5 and 1:2). In the case of 1:2 ratios, both DANI and DNA concentrations were halved (0.3 mM DNA/0.6 mM DANI) because of DANI solubility limit (1.5 mM). In the selected DNA/DANI ratio, the enzyme amount was optimized testing different concentrations from 1 to 0.02 U/mL. Samplings were done to record UV-Vis-NIR spectrum (300–800 nm) in order to follow polymerization reaction. Absorption values indicative of the polaron formation (900 nm) or of the presence of branched products (500 nm) were monitored during the time. Control reactions were carried out as described above. Polyaniline from DANI, namely pDANI, was recovered through precipitation with Acetone (1:1 v/v) and dried in SavantTM SPD121P SpeedVacTM (Thermo Fisher Scientific) at 37°C for 30 min for further characterization.

Aniline consumption during the polymerization was quantified following the protocol previously reported by Junker *et al* [30]. This protocol, with some modifications, was also used to determine DANI concentration during the polymerization. The UV-Vis spectrum of supernatant, after acetonitrile precipitation, was recorded and the specific absorbance at $\lambda = 286$ nm was used to evaluate DANI concentration by using $\epsilon_{286} = 715 \pm 21$ M⁻¹ cm⁻¹ empirically calculated from calibration curve. The activity of TvB in the reaction mixture was measured by using DMP as substrate as described above.

2.5 | Fourier transformed infrared spectroscopy

Fourier transformed infrared spectroscopy (FTIR) spectra of polymerized polyanilines were recorded by the conventional procedure using KBr pellets on an iS10 FT-IR spectrophotometer (Nicolet).

2.6 | MS analyses

Positive reflectron MALDI MS spectra were recorded using a MALDI-TOF 5800 (Applied Biosystems, Framingham, MA, USA). Different matrices as well as different polymer solvents (DMSO and THF) were tested. The MALDI matrices were prepared by dissolving 10 mg/mL α -Cyano-4-hydroxycinnamic acid (70 :30 v/v) tetrahydrofuran, and 50 mM citric acid (Sigma-aldrich), 2,5-dihydroxybenzoic acid (DHB) 10 mg/mL 70% tetrahydrofuran and 30% formic acid 0.1% (sigma -aldrich) and 10 mg/mL (50:50) water 0.1% formic acid and trifluoroacetic acid (Sigma-aldrich) for

positive ionization mode. Typically, 1 μL of matrix was applied to the metallic sample plate and 1 μL of analyte was then added. Samples were prepared by mixing a solution of the polymer (100 mg in 100 μL THF) with a solution of the matrix used, 1:1 vol/vol. The final solution was then deposited on a stainless-steel sample holder and the solvent was evaporated with an air current. When no matrix was added, polymer sample was directly added to MALDI plate. The analyses were performed, setting the instrument in the reflector mode. A laser pulse voltage power of 3500 V was applied to improve the detection of high molecular weight compounds in the MS spectra. A total of 5000 laser shots at 337 nm were summed for each spectrum. Raw data were analysed by using the Data Explorer Software (version 4.9, build 115; Applied Biosystems, Framingham, MA, USA) and reported as monoisotopic masses.

2.7 | Stereomicroscope (SM) and SEM analyses

Morphological and textural analyses of samples were carried out by means of Zeiss Axio Zoom V16 motorized microscope for large fields (Microscopy Center at DSC, Università degli Studi di Napoli Federico II) and by means SEM JEOL-JSM 5310 (CISAG laboratory, Università degli Studi di Napoli Federico II).

The microscope configuration for this study is with objective Apo Z 1.5X/0.37 FWD 30 mm. In particular, the images of details were acquired with maximum magnification of 168x, field of view of 1.4 mm, resolution of 0.4 μm and depth of field of 4 μm . The acquisition of images was carried out with AxioCam ICC5 (D) microscopes camera and with Zeiss Axiovision 4 software with Z Stack and Extended Focus modules.

Detailed morphological analyses of samples were performed with a SEM, operating at 15 kV, and equipped with energy dispersive X-Ray spectroscopy (EDS); data were processed with INCA version 4.08 (Oxford Instruments, 2006). Only DNA samples were metalized with gold, using a sputter coater. For each (PANI and pDANI) sample ten analytical points for different texture and morphologies were determined.

2.8 | Electrochemical impedance spectroscopy

The conductivity was measured by EIS and the comparison with the known value for PANI-ES provided a relative measurement of resistivity for PANI and pDANI. To this end, samples of synthesized PANI, Pdani, and commercial PANI-ES were resuspended in DMSO at 4% (w/v) final concentration. Electrochemical experiments were performed with a potentiostat/galvanostat, and impedance analyzer PALMSENS (Utrecht, Netherlands) model PalmSens3

controlled by a computer through the PSTRACE version 5 software. All electrochemical measurements were carried out in a conventional screen-printed electrode having gold as material deposited onto working electrode and in PBS buffer (pH = 7.4). EIS results were obtained by fixing the potential at 0.8 V and changing the frequency range from 0.1 to 10 000 Hz and using an amplitude perturbation of 5 mV. The Electrochemical Impedance Spectroscopy (EIS) spectrum Analyser software, supplied with the instrument, was used for fitting EIS data to the electrical equivalent circuit, in order to obtain the fit-component parameters values. All experiments were carried out at room temperature. The gold electrode surface was electrochemically cleaned in 0.1 M H_2SO_4 by potential scanning between -0.3 and 1.6 V at a scan rate of 0.1 V/s until a reproducible cyclic voltammogram was obtained. The electrode was rinsed with a copious amount of Milli-Q water and, after that, it was cycled from -0.6 to 0.8 V at 0.05 V/s scan rate in 10 mM for 10 scans. Then the electrode was rinsed with Milli-Q water and it was ready to use.

3 | RESULTS AND DISCUSSION

3.1 | Biocatalyst selection

The efficiency of different fungal laccases in aniline oxidation was tested, searching among high-redox potential enzymes, in order to overcome the high potential barrier for aniline oxidation at acidic pH ($E^\circ + 1.05$ V) [7]. Two native fungal laccases (POXC, TvB) and two recombinant ones (rPOXA1b, and its variant 1H6C [29]), were tested. POXC is a canonical laccase, the most abundantly isoenzyme produced by *P. ostreatus*, successfully applied to dye decolorization [31]. TvB, is a *T. versicolor* laccase, characterized for its activity against hydroxyphenylurea derivatives whose crystal structure with 2,5 xylidine was solved [23]. rPOXA1b is a *P. ostreatus* laccases exhibiting stability and activity in a wide range of pHs (3–9) and temperatures (25–65°C), already exploited in different application, recombinantly produced in a consolidated and cost-effective process [32]. 1H6C is an improved variant derived from POXA1b engineering, characterized by a higher redox potential than the wild type [29].

Despite a disadvantageous ΔE° between enzyme and substrate, all the tested laccases proved to be able to oxidize aniline, although with different efficiency (Figure 1). This result further highlights how laccase reactivity is an intricate multidimensional problem [33] in which the Electron Transfer driving force is an important factor depending on not only $T1 E^\circ$, but also on the right pose of the substrate within the binding pocket. Particularly relevant is the difference observed between rPOXA1b and 1H6C, where the latter enzyme, although characterized by a higher redox potential with respect to the wild type, displayed the worst performance

FIGURE 1 Comparison of laccase performances in aniline oxidation

in aniline oxidation. Among the tested enzymes, TvB showed the best performance providing the highest reactivity ($\Delta A_{410\text{nm}}/30\text{ min} = 3.2$) towards aniline. Based on these results, TvB laccase was selected for further investigation on DNA-assisted synthesis of the emeraldine salt of polyaniline.

3.2 | Laccase-catalyzed polyaniline synthesis

The polymerization reaction was investigated analysing the effect of different factors such as template/substrate ratio, time of reaction and enzyme concentration. Different DNA/aniline ratios were tested. A concentration of at least 1.5 mM of aniline is required to observe enzyme reactivity. The absorbance spectra were recorded at 2, 5, and 24 h to follow PANI polymerization (Figure 2). Soluble green PANI was obtained at 1:1; 1:2.5, and 1:5 DNA/aniline ratios by using the minimum amount of enzyme tested (1 U/mL of TvB) (Figure 2). Increasing enzyme concentration to 5 and 10 U/mL did not affect the rate of the process. When no template was used, or a high excess of aniline was adopted, only the formation of a black pernigraniline was observable, endorsing the presence of DNA has a significant influence on the success of the polymerization reaction.

UV-visible spectra of the green PANI synthesized at different template/substrate ratios displayed basically the same shape, being characterized by the absorption bands typical for emeraldine salt: at around 300 nm corresponding to benzenoid diamine units ($\pi - \pi^*$); at around 400 nm characteristic of the semiquinoid radical cation (corresponding to polaron- π^* benzenoid transition), and a broad peak with a maximum at 730 nm, the distinctive signal of doped PANI due to π -polaron electronic transition [34]. The low absorption at around 500 nm is indicative of the absence of branched structures in the polymer chain. During the time, the absorbance in

the entire spectra increased, indicative of the accumulation of the polymeric emeraldine salt. Interestingly, pronounced turbidity of solution was observed, followed by complex precipitation, after 24 h in the case of 1:5 ratio, and 36 h in case of 1:2.5 ratios. It can be inferred that complex solubility is influenced by the presence of free DNA phosphate groups [7]. As a fact, the lower is the aniline amount used, the lower is the polymerization extent achieved, leading to delayed precipitation.

The optimal conditions for the polymerization were selected on the basis of the following criteria: (i) high A_{730}/A_{500} ratio, indicative of the formation of low-branched product; (ii) high absorbance at 400 nm, indicating polaron band π^* transition; (iii) high absorbance of the entire spectra, indicating high reaction yields [11,30,35,36]. Based on spectra analysis, the optimal reaction condition was 1:5 DNA/aniline ratios, 24 h reaction in which the water-soluble product is characterized by a low degree of branching ($A_{730}/A_{500} \approx 4$).

In the applied conditions for PANI synthesis, TvB retains 98% of its activity after 24 h and 90% of aniline was consumed after the first hour of reaction. At the end of the reaction, the final yield of the product, recovered after acetone precipitation, is about 80%, giving 2.2 mg of product/mL of reaction. TvB demonstrated to be very efficient considering that the amount of enzyme used in this study is comparable to that reported by de Salas *et al.* [19], but significantly lower than that reported by other authors [11,36].

3.3 | Laccase-catalyzed polyaniline synthesis from DANI

Synthesis of polyaniline was also carried out starting from a less toxic substrate such as DANI, whose E° is lower than that

FIGURE 2 UV/Vis spectra recorded during laccase catalyzed oxidation of aniline monomer at different DNA/aniline ratios (A, B, C). Standard deviations among three replicates were less than 5%. The inset in each panel shows the color of the product at the latest time

of aniline [37,38]. Furthermore, only few papers have reported polyaniline synthesis from DANI, due to its low water solubility [3].

Different DNA/DANI ratios were tested, taking into account the lower DANI solubility with respect to aniline. As observed when aniline monomer was used, only black pernigraniline was produced in the absence of template. TvB

reactivity was measured in the range 0.6–0.9 mM of substrate. The UV-vis spectra recorded for the different tested conditions in the presence of the dimeric substrate displayed a different shape in comparison with those of PANI products (Figure 3). As a fact, the maximum absorption peak relative to polaron formation is centred at around 900 nm. The latter is red-shifted with respect to the corresponding

FIGURE 3 UV/Vis spectra recorded during laccase catalyzed oxidation of aniline dimer at different DNA/aniline ratios (A, C, D) and enzyme concentrations (A, B). Standard deviations among three replicates were less than 5%. The inset in each panel shows the color of the product at the latest time. When not specified, the reaction was carried out with 1 U/mL TvB laccase

broad peak in PANI spectra (730 nm), indicating a longer average conjugation length in the pDANI products [11,39]. Such difference also suggests the existence of a higher ratio of polaron/bipolaron species in pDANI with respect to PANI [34].

During the time, different spectrum shapes were observed at 1:1.5, and 1:2 ratios, probably relative to the formation of transient polymeric species as already observed in other reports. When a ratio in favour of DNA was used (1.3: 1) the highest amount of product was obtained in few minutes, although precipitation of the complex was observed within 1 h. To maintain the complex in a soluble form, the amount of enzyme was progressively reduced down to 0.02 U/mL. Slowing down the reaction rate allowed obtaining a water-soluble green polymer within 5 h of reaction using a very low amount of enzyme, while the product became insoluble after 10 h. Also, in this condition, the formation of intermediate polymeric products was observed.

In the optimal reaction conditions (1.3:1 DNA/DANI ratio, 0.02 U/mL TvB, 5 h) pDANI showed a high A_{914}/A_{500} ratio (~ 5) indicating low branching and therefore good quality of the polymer. Measurement of DANI consumption indicated that in the optimized conditions about 98% of DANI was converted within 10 min. The final yield of the product, recovered after acetone precipitation, is about 90%, corresponding to 0.5 mg/mL of reaction. Comparing the processes for PANI and pDANI synthesis, DANI polymerization occurred faster, required a fifty-fold lower amount of catalyst and achieved higher conversion yield. In addition, pDANI is characterized by extended conjugation as indicated by the absorption at $\lambda > 750$ nm, a signature for the presence of

linear *para*-linked conductive polymer [34]. Furthermore, pDANI is endowed with lower branching as indicated by the higher A_{914}/A_{500} ratio with respect to the corresponding A_{730}/A_{500} ratio for PANI.

3.4 | Characterization of polymerized products PANI and pDANI

3.4.1 | FTIR

The purified PANI and pDANI products were analyzed by FTIR spectroscopy. Both products exhibited typical FTIR bands of emeraldine form (Table 1) [18,20] even if some differences between the two polymers were found, as already observed by Junker and co-workers [9]. Interestingly, the 1070 cm^{-1} IR band characteristic of emeraldine salt form of PANI was present in both polymers (protonated NH^+), while only pDANI showed the presence of the 1405 cm^{-1} band, related to C-N^+ [40]. Moreover, pDANI displayed bands attributed to para substitutions in benzene ring whilst meta substitutions were present in PANI. These analyses are consistent with the absorbance spectra recorded after polymerization. Both polymers showed the presence of bands assigned to phosphate groups of DNA (2924 and 2854 cm^{-1}), while bands at 1730 cm^{-1} relatively to C=O in DNA and at 670 cm^{-1} of P-O-C appeared only in pDANI that is characterized by a higher DNA/DANI ratio.

3.4.2 | MALDI analysis

MALDI TOF-MS was used to further characterize the polymers. Different matrices and solvents were tested in order to verify the compatibility of the solvent-sample-matrix

TABLE 1 FTIR analysis of polymeric products

IR vibrational units	Wave number (cm ⁻¹)	
	PANI	pDANI
P–OH of phosphate group in the DNA chain	2924, 2854	2924, 2854
C=O double band in DNA base pairs		1730
asymmetric stretching of P–O–C		675
N–H stretching vibrations	3440	3440
C–H stretching vibrations in CH ₃	2961, 2921	2961, 2921
C=N stretching vibrations of quinoid ring	1650	1650
C=C stretching vibrations of benzoid ring	1515, 1496, 1450	1515, 1496, 1450
C=C stretching vibrations of quinoid ring		1597
C–N ⁺ stretching vibrations		1405
C–N stretching vibrations in aromatic primary amine	1255	1255
Quinoid ring –NH ⁺ – benzoid ring stretching vibrations	1070	1070
C–H out of plane bending vibrations	971	995, 971, 909
Meta substitutions, 1,3 di-substitution in benzene ring	873, 692	
Para substitutions, 1,4 di-substitution in benzene ring		830, 554

system and optimize the quality of mass spectra. No peaks were detected when the analysis was performed on both PANI and pDANI samples in the absence of matrix. The best results were obtained by using THF as solvent, and DHB as matrix. The mass spectrum of PANI revealed the presence of a complex mixture (Supporting Information Figure 1A). Polymeric species ranging from 400 to 900 Da could be observed and were tentatively assigned to oligomers of 5 to 9 repeating units (Δ = about 92 Da). This polymerization degree is lower than that reported for other enzyme-catalysed polyanilines [19,36]. The MALDI spectrum is very crowded and each oligomer showed the presence of different peak clusters corresponding to its various redox states and different end groups [41]. As an example, a mass increment of about 15 Da corresponding to polymeric species with loss of -NH_2 , may be the result of fragmentation of the sample in the desorption/ionization process, as reported elsewhere [42–45].

As for pDANI, the spectra showed the presence of polymeric species in the range from 700 to 1600 Da, with peaks belonging to two families with different abundance (Supporting Information Figure 1B). The major one showed peaks corresponding to oligomers ranging from 4 to 9 repeating units (Δ = 182 Da). Oligomers belonging to the second family, (showing the same repeating unit of 182 Da) differ from the species of the most abundant one for a phenylic moiety (Δ = 92 Da), as a consequence of polymer rearrangements. As for PANI spectra, satellite peaks are observed for each oligomer [42,43]. Oligomer length obtained for pDANI (8–18 in terms of aniline subunits) is in accordance with data reported by Shumakovich and De Salas [19,36] and longer than that observed for PANI.

3.4.3 | Morphological analyses

The morphology of purified products was analyzed by SM and SEM. SM analyses of PANI sample revealed two different textures, principally linked macroscopically to their chromatic change. The aggregates with dark green color (A) appeared rather porous and hard, instead the aggregates with bright green color (B) seemed more compact but light and fluffy (Supporting Information Figure 2A). Variable textures were also observed in pDANI, in some cases compact (A), porous (B) and, in other cases, frayed (C) (Supporting Information Figure 3A).

Despite the differences of color, texture aggregates, and morphology, the chemical qualitative analyses, carried out with energy dispersive X-Ray spectroscopy (EDS), showed the same compositions of materials, which contain P, O and C, as expected by the presence of the DNA template (Supporting Information Figures 2B and B).

The morphology of the polymeric products was deepened through SEM analyses and compared with that of DNA samples processed and recovered as the polymers, but in the absence of substrates.

DNA sample revealed a complex texture, characterized by the co-presence of flat and uniform sheets and irregularly holed structures (Figure 4). PANI is organized into irregular and flexible lamellae layered forming sub-circular cavities, locally covered by green light aggregates composed by smaller elements with variable shapes. As for pDANI, the micro-structures composing the polymers also formed sub-circular cavities enveloped by lamellae of different sizes (Figure 4, pDANI 1 and 2) located in different areas of the sample (Supporting Information Figures 3A), probably linked to the DNA texture they covered. Thus, DNA seems

FIGURE 4 SEM micrographs of DNA, PANI, and pDANI samples at different magnifications (general, upper row; and detailed, lower row). pDANI 1 and 2 show small and large lamellae, respectively

to work as hard template for both enzymatically synthesized polymers, since all analyzed samples display the same structure characterized by repeated holed blocks. The observed morphology of the DNA/polymer complexes may be the result of a hierarchical structuring on DNA platform, which confers them a semi-ordered organization.

A wide variety of micro- and nanoscale structures have been described for polyaniline synthesized by different processes, being the morphology dependent on the applied oxidative routes as well as on seemingly innocuous changes, such as the agitation occurring during polymerization [5]. Nanowire structures have been observed in the case of HRP catalysed aniline oxidation on Si surfaces with DNA-templates [14]. On the other hand, different morphologies, *i.e.* PANI-DNA micro/nano-wires or nanofibers, have been reported during chemical aniline oxidation, depending on DNA/aniline ratio [20]. The polyaniline microstructure obtained in this work by the combination of laccase as biocatalyst and DNA as template, adds up to the other unusual structures already reported for this polymer, confirming polyaniline propensity to be shaped depending on process conditions.

3.4.4 | Electrochemical impedance spectroscopy

The electrical properties of PANI and pDANI were assessed relatively to the commercial PANI-ES, whose conductivity is known. Since dealing with conductive polymers, the commonly used $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ solution gave rise to a narrow impedance spectrum with barely appreciable differences among the species. On the contrary, by adopting PBS 1X solution, we were able to measure the spectra shown in Figure 5, from which impedance of the order of $\text{k}\Omega$ s are observable as a consequence of the tiny values of current

FIGURE 5 Nyquist plots for PANI-ES, PANI, and pDANI. The lines express fitting results for corresponding EIS data, and the inset gives the equivalent circuit which includes the impedance characteristic of the electrodes described by the charge transfer resistance (R_{ct}), solution resistance (R_s) and double layer capacitance (C_{dl})

brought by PBS solution [46,47]. Due to its poor conductive properties, DNA is not included in Figure 5 since its impedance is very large [48] and, hence, out of scale. The polymers were adsorbed onto the gold surfaces of screen-printed electrodes by drop-casting 5 drops (5 nL each) of polymer solutions all having the same concentration. Such a procedure led to a visible uniform coloration of the surface and also to a saturation of the EIS spectrum. Since the polymers do not aggregate, these features can be ascribed to the formation of a single layer of polymers resulting from their interaction with gold.

The data in Figure 5 were analyzed using the Z-view software adopting the Randles-type circuit (inset of Figure 5), which includes the impedance characteristic of the

TABLE 2 EIS characterization of polymers

Sample	R_{ct} (Ω)	ρ (S/cm)
Commercial PANI	$7441 \pm 5\%$	3 (reference)
PANI	$3857 \pm 5\%$	$5.8 \pm 5\%$
pDANI	$3446 \pm 5\%$	$6.5 \pm 5\%$
DNA	$5659 \times 10^5 \pm 7\%$	$4 \times 10^{-5} \pm 7\%$

electrodes described by the charge transfer resistance (R_{ct}), solution resistance (R_s) and double layer capacitance (C_{dl}). The charge transfer resistance (R_{ct}) is a measure of the rate of electron transfer between the redox species and the electrode during the electrochemical reaction course, whereas the solution resistance (R_s) represents the solution-phase interference that primarily arises from the electrolyte resistance [49,50].

The EIS spectra are well fitted by the Randles-type circuit and the resulting R_{ct} are reported in Table 2 with the errors as low as 5% worked out by simulating the circuit with PSTrace 5 software (PalmSens BV) based on Levenberg-Marquardt algorithm. Since all the polymer layers onto gold have the same geometry, the resistivity of PANI and pDANI can be retrieved by the known value for commercial PANI (3 S/cm, Sigma-Aldrich technical sheet). The analysis is reported in Table 2, which clearly shows that both polymers reach impressive conductivity of ~ 32 S/cm, values attained only in strongly acidic media for synthesized polyanilines [51–53]. These considerable conductivities confirm that DNA, besides acting as template, plays a pivotal role in polyaniline doping [20]. More interestingly, the obtained values are significantly higher than those measured for other DNA-PANI complexes ($\sim 10^{-2}$ S/cm) synthesized via both chemical and enzymatic routes with a lower amount of DNA [15,20]. The slight difference of conductivity between PANI and pDANI is consistent with the UV/Vis and FTIR spectroscopic characterization. It can be envisaged that the combination of TvB laccase as oxidant and DNA as template and dopant, has a dramatic effect on polymer conductivity.

4 | CONCLUDING REMARKS

The work presented here, developed an environmentally-friendly method to produce conductive polyaniline, by using TvB laccase as oxidant and DNA as template/dopant. To the best of our knowledge, this is the first work reporting laccase-catalyzed polyaniline/DNA complexes. Tuning of reaction conditions allowed controlling the process and obtaining water-soluble polyaniline in its early phases. When the less toxic aniline dimer was used as substrate, the polymerization reaction was faster and gave less-branched polymer, as confirmed by absorbance spectra and FTIR analysis.

SEM images of both complexes proved the role of DNA as hard template, being the polymer characterized by the presence of repetitive sub-cavities microstructures resembling the holed structure that DNA acquires during synthetic processing.

Both TvB synthesized polymers display extraordinary conductive properties if compared with most of polyanilines obtained by conventional methods. The observed semi-ordered structures may render the polyanilines more organized, and consequently highly conductive. The template action combined with DNA-doping effect seems to magnify polymer properties leading to an unexpected value of conductivity.

Although some serendipity cannot be excluded, the herein developed combination of laccase and DNA represents a breakthrough in the green synthesis of conductive materials.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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