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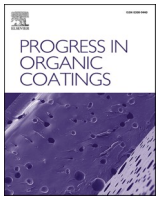

Laccase-mediated dopamine polymerization: a bioinspired and sustainable coating technology

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ABSTRACT

Polydopamine (PDA) has emerged as a widely used coating for various materials due to its unique properties such as robustness, biocompatibility, and antioxidant and photothermal activities. PDA coatings are usually prepared through autoxidation of dopamine under alkaline conditions, but this approach is generally time-consuming and requires high concentrations of the starting monomer. To overcome these drawbacks, enzymatic catalysis has emerged as a novel approach, allowing also to work in a wider range of pH values. In this work, the laccase-assisted dopamine polymerization and PDA film formation have been systematically investigated and optimized by varying reaction parameters, including dopamine and laccase concentrations as well as stirring speed and deposition time. The best results have been obtained at a low dopamine concentration (2.5 mM) in the presence of 0.25 mg/mL of laccase at pH 5.5, and under high stirring (1250 rpm). Under these conditions, laccase-assisted PDA film deposition resulted in significantly improved coating formation than that obtained through traditional autoxidation at pH 8.5 or sodium periodate-induced oxidation at pH 5.5 by approximately 5- and 1.6-fold, respectively. Insights into the chemical structure and morphological features of the PDA coatings were obtained by liquid chromatography–ultraviolet detection–mass spectrometry (LC-UV-MS), atomic force microscopy (AFM), kelvin-probe force microscopy (KPFM) and attenuated total reflectance-Fourier-transform infrared (ATR-FTIR) spectroscopy analysis. In addition, water contact angle (WCA) measurements were performed. These results provide a significant step forward for the obtainment of organic coatings under mild and sustainable conditions opening new possibilities for future applications in PDA-based functional materials.

1. Introduction

Because of its robustness, universal adhesion properties, simple coating procedures, biocompatibility, and functional properties [1–3], polydopamine (PDA), a mussel-inspired melanin-type polymer deriving from oxidation of the catecholamine dopamine [4], has been widely exploited over decades for surface functionalization in different fields. These include applications in biomedical engineering, drug delivery, medical implants, antibacterial coatings, and cosmetics [5–9]. Despite increasing insights into the unique underwater adhesion properties of PDA on both hydrophilic and hydrophobic materials [10], there still remain a number of issues that should be addressed. This would allow to expand the scope and technological impact of this versatile multifunctional material. Current limitations may include long deposition time (up to 24 h), the need for high dopamine concentrations ($\geq$ 5 mM) which usually produce an undesirable PDA precipitate, the use of an alkaline pH ($>$ 8.5), which might not be suitable for alkaline sensitive materials or biological systems, and difficulties in controlling the film thickness and properties [10–13].

A recently pursued approach to overcome these limitations relies on the use of enzymes as oxidation catalysts. Enzymes can, in fact, provide several remarkable features such as short reaction times, substrate specificity, as well as regio- and stereoselectivity. For instance, tyrosinase-mediated oxidation has been proposed as a simple and

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efficient strategy for preparing nanometre-thick and uniform PDA films on various surfaces, with diverse functionalities depending on the starting monomer [14–16]. Laccase-catalyzed polymerization of dopamine at acidic pH has been recently reported as a convenient method for surface functionalization and coatings, leading to good-quality films on various substrates, spanning from carbon nanotubes to cotton and bamboo fibres [17–20].

Laccase (EC 3.10.3.2) is a low-cost enzyme belonging to the oxidoreductase family. It can catalyze the oxidation of an extensive range of organic and inorganic substrates [21,22] by simply reducing molecular oxygen to water [23]. Laccase-mediated polymerizations may, therefore, represent greener and more efficient alternatives to conventional oxidations involving non-enzymatic oxidants [21,24,25]. However, despite a promising set of observations [24,25], the potential of laccase for PDA surface functionalization and coatings has not been systematically explored.

On this basis, the present work systematically investigated and optimized laccase-assisted dopamine polymerization and PDA film formation by varying reaction parameters, including dopamine and laccase concentrations, stirring speed, and deposition time. This assessment highlighted the possibility to implement high quality PDA coatings using significantly lower dopamine and laccase concentrations compared to what previously reported [25]. In addition, atomic force microscopy (AFM) and kelvin-probe force microscopy (KPFM) analysis of the coated surfaces revealed higher morphological homogeneity of the PDA films achieved by the use of laccase in comparison with those obtained by conventional (autoxidation at alkaline pH) and non-enzymatic oxidation at acidic pH.

2. Methods

2.1. Materials and methods

Dopamine hydrochloride, laccase (from *Trametes versicolor*, 1.4 U/mg), citric acid (>99% purity), disodium hydrogen phosphate dihydrate (>99% purity), and hydrochloric acid (37% *v*/v) were purchased from Sigma-Aldrich. Sodium periodate ($NaIO_4$) (>99% purity) was purchased from Analyticals Carlo Erba. Tris(hydroxymethyl)aminomethane (Tris) (>99% purity) was purchased from Pronalab. Cover glasses (22 × 22 mm, 0.12-0.16 mm thickness) and quartz slides (2 × 2 mm, 1.1 mm thickness) were purchased from AmScope.

UV−Vis spectra were recorded on a Jasco V-730 spectrophotometer.

LC-UV-MS analysis were performed on an Agilent ESI-TOF 1260/6230DA instrument coupled with a UV-Vis detector set at 254 nm or 280 nm, in positive or negative ion mode in the following conditions: nebulizer pressure 35 psig; drying gas (nitrogen) 10 L/min, 325 °C; capillary voltage 3500 V; fragmentor voltage 175 V. An Eclipse Plus C18 column, 150 × 4.6 mm, 5 μm, at a flow rate of 0.4 mL/min was used, with 0.1% formic acid (eluant A)–methanol (eluant B) under the following gradient conditions:

- from 10 to 80% B, 0–45 min, detection wavelength 280 nm (eluant I);
- 5 % B, 0–10 min; from 5 to 80% B, 10–47.5 min, detection wavelength 254 nm (eluant II).

The surface topography and surface electric potential of PDA films at the nanoscale were investigated by atomic force microscopy (AFM) and Kelvin-probe force microscopy (KPFM), respectively, using a Ntegra Aura Atomic Force Microscope (NT-MDT, Russia). Conductive Cr/Pt-coated Tap190E-G cantilevers (Budget sensors, Bulgaria) with a spring constant of 48 N/m and resonance frequency of 190 kHz were used. Topography measurements were performed in semi-contact mode at the fundamental resonance of the cantilever. Surface potential of the films was determined with a two-pass technique under a non-contact regime at the fundamental resonance of the cantilever.

Attenuated total reflectance-Fourier-transform infrared (ATR-FTIR) spectra were recorded on a Jasco FT/IR-4X instrument.

The wettability of the materials was evaluated by measuring the water contact angles (WCA) using an Attension Theta Optical Tensiometer. The analysis was carried out using the sessile drop technique at room temperature. All the measurements were carried out in triplicate. 4 μL of distilled water was dropped on the air side surface of the substrate.

Coating adhesion on glass slides was evaluated by means of a tape test, in which a standard adhesive was applied to the surface and then removed at once. The test has been performed on one sample per type of surface. The surface was then inspected by means of visible spectroscopy.

2.2. General procedure for substrate coating

For the enzymatic-oxidation process, laccase (0.05–1 mg/mL final concentration) was added to a dopamine hydrochloride solution (0.5–10 mM final concentration) in 0.15 M sodium citrate-phosphate buffer (pH 5.5) (final volume 20 mL). Glass or quartz slides were dipped into the reaction mixture immediately before laccase addition and left under stirring (150–1250 rpm) for different periods (2–24 h), before being extensively rinsed with distilled water, air-dried and analyzed by UV-Vis spectroscopy.

In other experiments, laccase (0.25 mg/mL) was added to a dopamine hydrochloride solution (1.25 or 2.5 mM final concentration) in 0.15 M sodium citrate phosphate buffer (pH 5.5) (final volume 20 mL). Glass or quartz substrates were dipped into the reaction mixture immediately before laccase addition and left under stirring (1250 rpm). After 6 h, an equal amount of dopamine hydrochloride was added to the reaction mixture, which was left under stirring for an additional 24 h; after that, the coated substrates were processed as above.

For the conventional coating procedure, the oxidation of dopamine (2.5 or 10 mM) was run in 0.5 M Tris buffer (pH 8.5), and the glass or quartz slides were dipped for 24 h in the reaction mixture and then treated and analyzed as described above. For the non-enzymatic coating at acidic pH, the oxidation reaction was run on 2.5 mM dopamine at pH 5.5 (0.15 M sodium citrate phosphate buffer) with the addition of 1 molar equivalent of sodium periodate. Glass or quartz slides were treated and analyzed as above.

When required, the reaction mixtures were periodically analyzed by LC-UV-MS (eluant I).

The optimized reaction conditions selected for the coating characterization are reported in Table 1.

2.3. PDA chemical degradation analysis

Dopamine oxidation was run in the presence of glass slides under the reaction conditions A-C (Table 1).

After 24 h, glasses were removed and extensively washed with distilled water and air-dried, whereas the reaction mixtures were centrifuged at 7000 rpm at 4°C, and the precipitate washed three times with water and lyophilized to collect PDA.

5 mg of each PDA sample was suspended in 1 M NaOH (1 mL) and treated with 1.5% (final concentration) H_2O_2 at room temperature and under vigorous stirring [26] under an argon atmosphere. After 24 h, 5% sodium metabisulfite (200 μL) was added, the pH was adjusted to 2 with 2 M HCl, and the mixture was filtered through a nylon membrane (13 mm, 0.45 μm) and analyzed by LC-UV-MS (eluant II).

Similarly, PDA-coated glasses were dipped in 1 M NaOH (2 mL) and treated with 1.5% (final concentration) H_2O_2 at room temperature and under an argon atmosphere. After 24 h, the mixtures were treated and analyzed as above. All the experiments were run in triplicate.

Table 1
Reaction conditions adopted for the preparation of PDA samples for chemical and morphological characterization.

Method	[Dopamine] (mM)	[Laccase] (mg/mL)	[$NaIO_4$] (mM)	pH	Stirring rate (rpm)	Time (h)
A	2.5	0.25	None	5.5 (0.15 M citrate/phosphate buffer)	1250	24
B	2.5	None	None	8.5 (0.5 M Tris buffer)	1250	24
C	2.5	None	2.5	5.5 (0.15 M citrate/phosphate buffer)	1250	24

3. Results and discussion

3.1. Laccase-mediated PDA film deposition

In an initial set of experiments, the ability of laccase to induce PDA film deposition was investigated by running the reaction at the conventional 10 mM dopamine concentration in citrate-phosphate buffer at pH 5.5 under stirring in air with 1 mg/mL (1.4 U/mL) of laccase, as described in a previous work [21]. For comparison, PDA film deposition was also achieved under conventional conditions, that is, by dopamine autoxidation in alkaline Tris buffer pH 8.5 [11,27]. Glass slides were dipped into the reaction mixtures, and the PDA film formation on the material surface was evaluated after 24 h. For the purpose of these experiments, the visible absorption determined by UV-Vis spectroscopic analysis of the glass substrates was taken as an index of film thickness to estimate the degree of PDA coating deposition [11]. Visible spectra and digital pictures of the PDA-coated glasses produced by auto- or enzymatic oxidation are shown in the Supplementary Material (cf. Fig. S1). Data showed similar absorption profiles for films produced in air at pH 8.5 or in the presence of laccase at pH 5.5, whereas, as expected, dopamine periodate autoxidation performed at pH 5.5 as a control was not effective in inducing a detectable film deposition. The potential of the laccase-mediated PDA film deposition approach is therefore already evident from this first result. The possibility of working in an acidic medium minimizes dopamine autoxidation and provides a stricter process control compared to conventional deposition at alkaline pH.

In subsequent experiments, the laccase-catalyzed PDA coating process was systematically investigated by varying several parameters, including dopamine and laccase concentration, stirring rate and time. Comparative analysis of film deposition, reported in Fig. 1, was conveniently carried out by measuring the absorbance values of the coated glass slides at 400 nm after 24 h dipping in the reaction mixture.

Firstly, keeping the laccase concentration constant at 1 mg/mL, the effect of initial dopamine concentration in the 0.5–10 mM range on PDA film formation was evaluated. All reactions were carried out in air at low stirring rate (150 rpm). As shown in Fig. 1A, laccase-mediated dopamine oxidation resulted in the deposition of films at all the concentrations explored, even at 0.5 mM, the lowest value so far reported for PDA film deposition using laccase as a catalyst [20,24,25]. In addition, it is worth underlining that in autoxidation experiments, at least 5 mM dopamine is required for significant film deposition [11,13,28]. This further adds to the advantages related to the laccase-mediated strategy for PDA surface coatings from a green, atom-economy perspective. Additionally, the data showed an increase in PDA coating going from 0.5 to 2.5 mM starting dopamine concentration, followed by a decrease when higher concentrations (5 and 10 mM) were used, in line with what was previously observed in the case of PDA film deposition by autoxidation on TiO_2 substrate [29]. This trend could be explained considering the strict relationship between concentration and aggregate/precipitate formation and the delicate balance between these two factors required for efficient PDA film deposition. When the concentration of dopamine increases, extensive polymer precipitation occurs, negatively affecting film deposition. In the present work, the highest extent of PDA coating was observed using dopamine at starting 2.5 mM concentration, which is still lower compared to that required by the generally used autoxidative protocols. At 5.0 mM, a rapid formation of insoluble aggregates was observed in the reaction mixture, which limited the availability in solution of dopamine intermediates necessary for controlled film growth on the substrate. This observation suggests a shift toward bulk precipitation rather than surface deposition. The increase in PDA coating

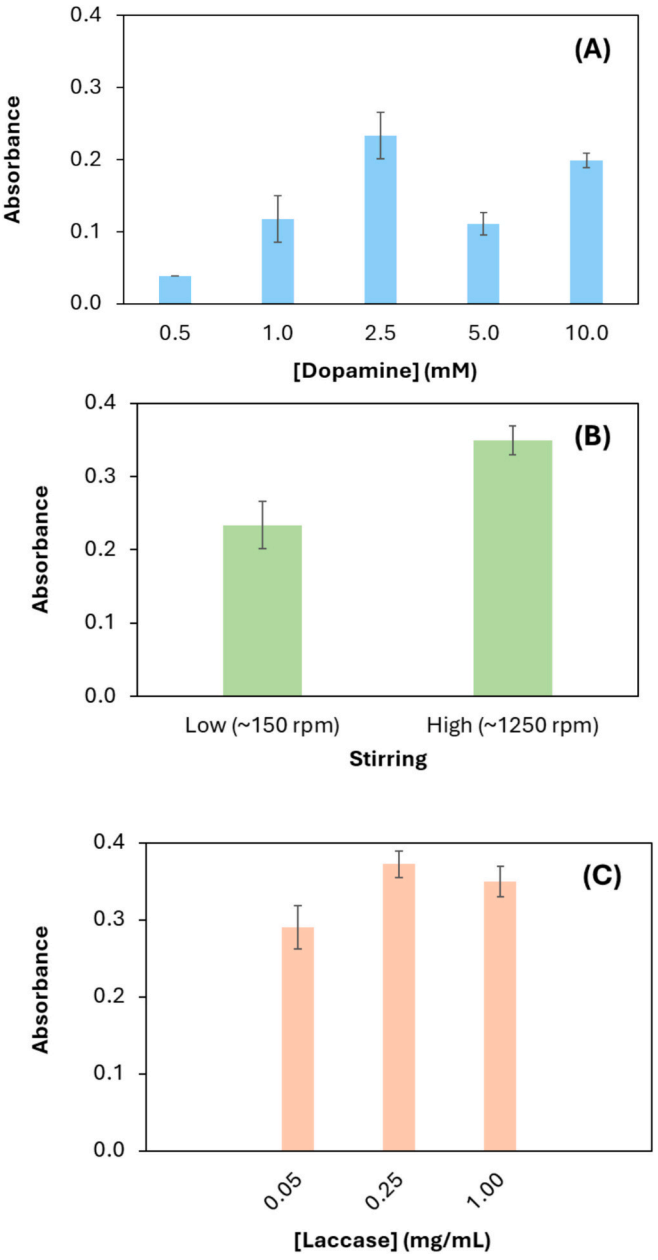

Fig. 1. Absorbance at 400 nm of the PDA-coated glasses prepared by varying: (A) dopamine concentration, keeping fixed the stirring rate (150 rpm) and the laccase concentration (1 mg/mL); (B) stirring rate, keeping fixed dopamine (2.5 mM) and laccase (1 mg/mL) concentrations; (C) laccase concentration, keeping fixed the stirring rate (1250 rpm) and dopamine concentration (2.5 mM). Reported are the mean ± SD values obtained by three independent experiments.

formation from 0.5 to 2.5 mM dopamine, followed by a decrease at 5 mM and then a second increase at 10 mM, suggests multiple competing processes during PDA film formation. At 5 mM local supersaturation of dopamine and its oxidized intermediates can lead to: (i) rapid polymerization in solution, forming PDA aggregates; (ii) depletion of reactive intermediates at the substrate surface due to their rapid consumption and hence reduced effective surface deposition. When dopamine concentration increases to 10 mM, although precipitation still occurs, the absolute amount of dopamine is high enough that sufficient quantities of intermediates remain near the substrate. This can result in increased coating formation, though it may be less uniform and less reproducible compared to what observed at lower dopamine concentrations. Additionally, partial sedimentation of aggregates onto the surface might falsely contribute to increased absorbance [13].

In subsequent experiments, at the optimal dopamine concentration of 2.5 mM, the effect of the stirring rate of the oxidation mixture on film deposition was evaluated. Fig. 1B shows the absorbance values at 400 nm of the glass slides coated under low (150 rpm) and high (1250 rpm) stirring rates. Film deposition was found to be slightly higher at high stirring speed. Increasing the agitation of the solution increases, in fact, the amount of oxygen in the solution, which is necessary for the enzymatic reaction, and hence promotes dopamine oxidation. However, increasing the speed above 1250 rpm is not recommended since the vortex created in the reaction mixture makes difficult for the dopamine oxidation products to come into contact with the glass. In any case, since the extent of dopamine polymerization under oxygen has been reported to be comparable to that achieved in air [25], the possibility to work under an oxygen flow was not considered due also to its high cost.

Lastly, laccase concentration was varied between 0.05 and 1 mg/mL, while dopamine concentration and agitation were maintained constant at 2.5 mM and 1250 rpm, respectively. As evident from Fig. 1C, PDA coating on glass slides was observed with all laccase concentrations explored, with the highest extent of film formation achieved already with 0.25 mg/mL of laccase. Of course, reducing the amount of laccase, with respect to the usually used concentration of 1 mg/mL [24,25], may be a valuable option in the perspective of reducing the cost of the process. It is therefore worth of noting that even 0.05 mg/mL of laccase was enough to induce PDA film deposition of the glass slides, turning thus the process more sustainable.

The time-evolution of the laccase-mediated PDA film deposition was also investigated by dipping glass slides for different times in a 2.5 mM dopamine solution undergoing oxidation in the presence of laccase (0.25 mg/mL). UV-Vis analysis showed that the highest extent of coating was reached after 24 h, but even after only 2 h film deposition was significant, with an absorbance value at 400 nm of the coated glass reaching approximately 67% of the value reached after 24 h. However, it is essential to remember that the amount of enzyme may affect the reaction kinetics. Thus, this parameter should be properly modulated depending on the desired process to be applied. Although the kinetics of the enzymatic process weren't studied in this work, further insights into enzyme efficiency and its role in reaction progression has been discussed in Section 3.3, where sequential dopamine addition was explored to optimize film deposition.

The optimal laccase-mediated dopamine oxidation conditions (Method A) were then applied to both autoxidation in Tris buffer at pH 8.5 (Method B), and non-enzymatic oxidation using sodium periodate as oxidizing agent [30] at pH 5.5 (Method C). All the reactions were run starting from 2.5 mM dopamine for 24 h using a stirring rate of 1250 rpm. Fig. 2 shows the absorption at 400 nm of the coated glasses. Notably, under these conditions, autoxidation at pH 8.5 led to a hardly detectable coating formation (the absorbance value at 400 nm was only 0.07± 0.02), whereas, compared to the periodate, laccase induced a 60% increase in PDA-film deposition.

Finally, to validate the obtained results, PDA-film deposition mediated by laccase under the optimal conditions was run using quartz slides as substrates. As reported in Fig. S2 in the Supplementary Material, glass and quartz coatings showed the same absorption profile in the visible region, suggesting that the parameters optimized for glass substrates can be applied to quartz, and likely other materials, without loss of PDA coating efficiency. This would confirm the universal adhesion properties of PDA even when film deposition is achieved under more sustainable conditions as in the present work. In the case of the quartz slides, it was possible to record the spectrum of the PDA-film in the UV region (Fig. 3). The film showed significant absorption in the 200–400 nm region, which was higher than that observed for PDA-coatings obtained by dopamine periodate-mediated oxidation and autoxidation, confirming the efficiency of the laccase-promoted PDA film deposition. Moreover, the tape test indicated a slightly more efficient coating adhesion for this latter. Indeed, after the test the coating was maintained for 97% in the case of Method A and for 93% in the case of Methods B and C (Fig. S3).

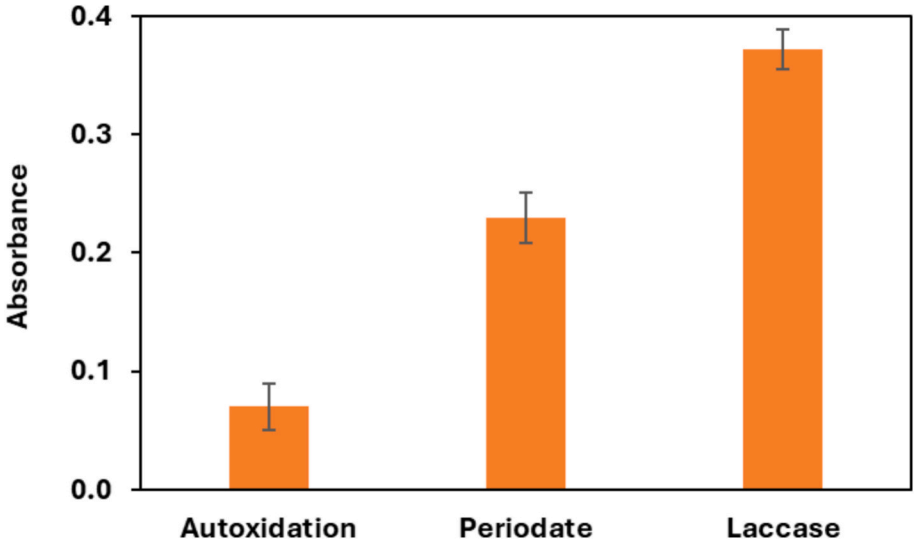

Fig. 2. Absorbance at 400 nm of the PDA-coated glasses obtained under autoxidation (Method B), non-enzymatic (Method C) and laccase (Method A)-mediated oxidation. Reported are the mean ± SD values obtained by three separate experiments.

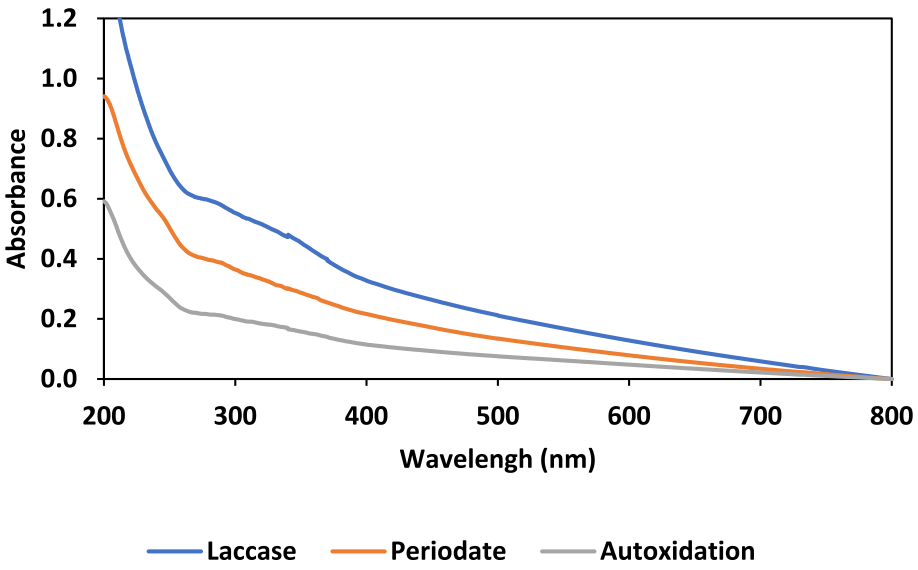

Fig. 3. UV-Vis absorption spectra of PDA coated quartz slides.

3.2. Chemical and morphological insight into the PDA films obtained by laccase-promoted dopamine oxidation

AFM was used to investigate the topography and surface roughness of the PDA films. Representative images are displayed in Fig. 4 and Fig. S4 in the Supplementary Material. Glass slides coated with PDA using laccase under the optimal conditions were compared to those obtained by 2.5 mM dopamine autoxidation in Tris buffer at alkaline pH and by periodate-induced oxidation. The topography of all deposited films appeared granular, with root mean square (RMS) roughness values (defined as the standard deviation of the height values over the scan area) ranging from *ca.* 60 to 112 (Table 2). In addition to the granular features, some larger areas with lower RMS, especially in the case of laccase-catalyzed film deposition, were apparent, suggesting the high-

Table 2
Surface roughness (at different scales) and surface potential average values.

Surface material	RMS, nm (4 × 4 μm^2)	RMS, nm (0.4 × 0.4 μm^2)	Surface potential, V
Glass	2.4	1.6	0.37 ± 0.01
PDA-TRIS (Method B)	60	5.8	0.39 ± 0.01
PDA-LACC (Method A)	99	3.5	0.45 ± 0.03
PDA-PERIODATE (Method C)	112	4.0	0.44 ± 0.03

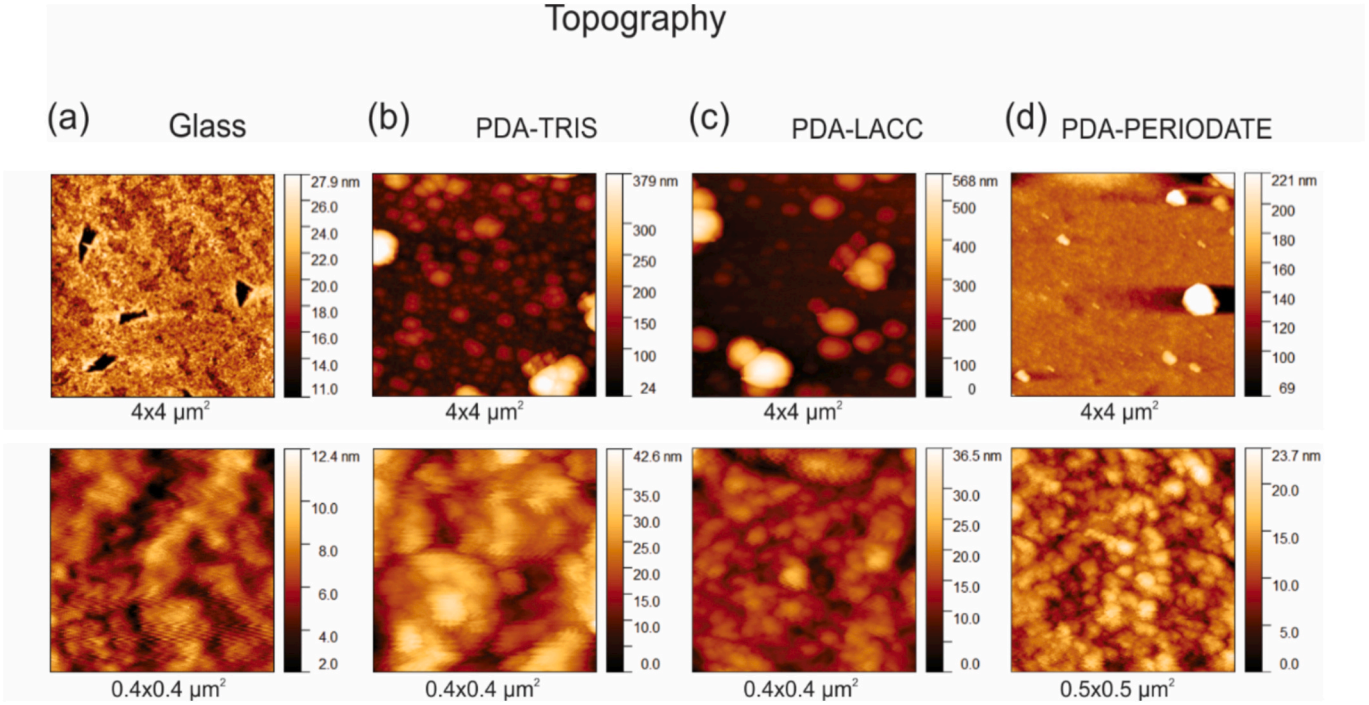

Fig. 4. AFM topography (semi-contact mode) of uncoated glass slide (a) and PDA-coated glass slides obtained by dopamine autoxidation (PDA-TRIS, Method B) (b), laccase-mediated oxidation (PDA-LACC, Method A) (c) and periodate-induced oxidation (PDA-PERIODATE, Method C) (d) at different scales.

quality of these latter coatings. In addition, a direct measurement approach of surface potential by using KPFM, based on a standard AFM tapping mode, was performed (Table 2 and Fig. S4 in the Supplementary Material). The results showed some differences in surface potential of PDA coatings obtained by autoxidation and laccase- or periodate-induced oxidation of dopamine, as expected based on the different pH conditions adopted for film deposition.

WCA measurements indicated a slightly more hydrophilic character for the coatings prepared with Method A (34° ± 3) and Method C (19° ± 4) compared to PDA coating produced by autoxidation (42° ± 12, Method B). This result was not so unexpected considering the higher number of protonated amino groups in the coatings obtained under acidic conditions.

ATR-FTIR spectra of PDA samples collected as precipitates from the laccase-catalyzed (Method A) and periodate-induced (Method C) dopamine oxidation mixtures as well as from the dopamine autoxidation mixture (Method B) are displayed in Fig. S5. Notably, the spectrum of the PDA sample deriving from autoxidation looked different from that of the PDA samples obtained under acidic conditions. Indeed, these latter where characterized by the presence, among others, of a signal at 1715 cm^{-1} likely due to C═O stretching in quinone species [31], which was lacking in the spectrum of the PDA sample obtained by aerial oxidation. This observation is in line with what previously reported [30]. Although less evident due to the low signal-to-noise ratio, this band was also present in the spectra of both laccase- and periodate-induced coatings (Fig. S5).

To obtain information about the main structural units present in the PDA sample obtained by using laccase, a chemical degradation procedure commonly used for the characterization of melanin-type pigments was applied. This involves treatment with alkaline hydrogen peroxide followed by LC-UV-MS quantitation of diagnostic markers of indole units, namely pyrrole-2,3-dicarboxylic acid (PDCA) and pyrrole-2,3,5-tricarboxylic acid (PTCA) [26,28]. These markers originate from 5,6-dihydroxyindole (DHI) units derived from the oxidative cyclization of dopamine. PDCA is a marker of indole units unsubstituted at C-2, chiefly terminal units, whereas PTCA is related to indole units linked through the 2-position, according to the prevalent mode of coupling of DHI in solution (Fig. 5) [32,33]. Thus, the absolute values of PDCA and PTCA concentrations are related to the extent of dopamine cyclization during the oxidation process, whereas the PTCA/PDCA ratio may be considered as an index of the involvement of indole units in dopamine polymerization [34].

On this basis, PTCA/PDCA ratios for PDA samples collected as precipitates from dopamine oxidation mixtures (Methods A-C). In addition, PDA films obtained under the same enzymatic, non-enzymatic and aerial oxidation conditions were subjected to the same degradation procedure.

Regarding the PDA solid samples, PDCA and PTCA were found to be present in all the chemical degradation mixtures (cf. Fig. S6 in the Supplementary Material for a representative sample), although in different amounts. Indeed, higher quantities (up to 3.5-fold) were observed in the case of samples deriving from oxidation run at pH 5.5, indicating a higher involvement of indole units in the dopamine polymerization process compared to the autoxidation conditions.

PDA samples prepared by laccase and periodate oxidation exhibited similar PTCA/PDCA ratios (Table 3), which were lower compared to that determined for the standard PDA sample prepared in Tris buffer at alkaline pH. This would suggest first that the oxidative chemistry of dopamine is more strongly affected by pH rather than by the type of oxidant and that a lower number of 2-linked DHI units is involved in the acidic polymerization process. This result was expected considering that DHI polymerization through the 2-position is strongly favoured in alkaline solutions [32,33,35,36]. Notably, however, PDA coatings deriving from laccase oxidation displayed a PTCA/PDCA ratio of 0.49 *vs* a value of 0.21 observed in the case of the periodate-mediated PDA coating. This indicates a higher involvement of DHI-related units in the laccase-mediated PDA-film deposition process, although still lower than

Table 3
PDCA and PTCA values by degradation of PDA samples. Reported are the mean values of two separate experiments (SD ≤ 10%).

	PTCA/PDCA	
PDA preparation conditions	Solid sample	Coating
Enzymatic oxidation (Method A)	0.29	0.49
Non-enzymatic (periodate) oxidation (Method C)	0.27	0.21
Autoxidation (Method B)	0.76	0.99

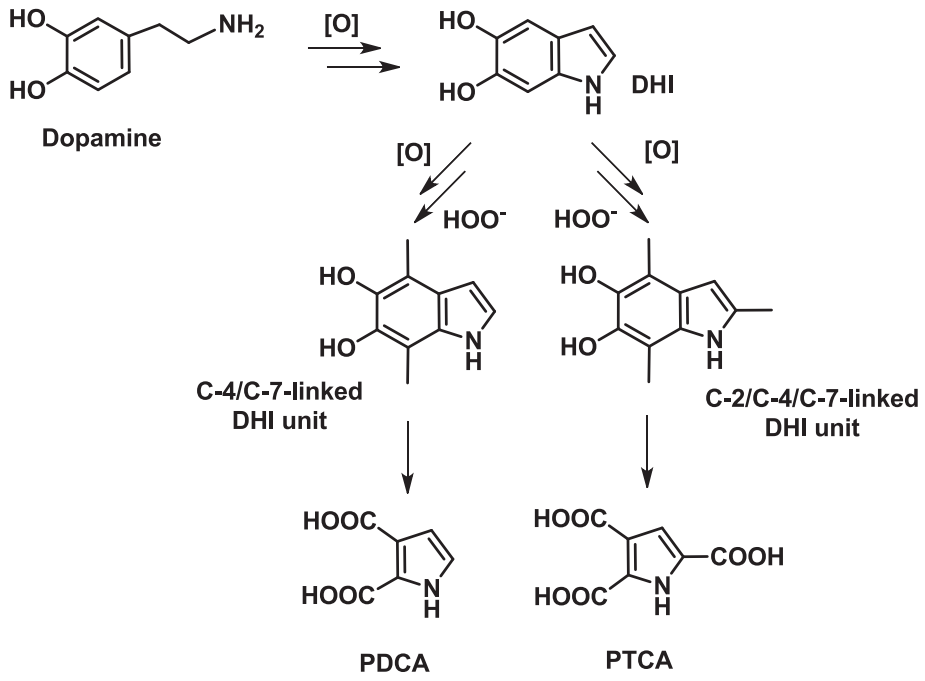

Fig. 5. Proposed origin of PDCA and PTCA by chemical degradation of PDA samples.

in the case of the PDA films prepared in Tris buffer (Table 3). Therefore, the chemical composition of the films and the species that adhere to the surface seem to strictly depend on the type of oxidizing system used. In any case, a higher PTCA/PDCA ratio was generally observed for the coatings compared to the PDA powders. This result is in line with the well-recognized key role of cation-π interactions in the adhesion properties of PDA [37]. Indeed, as mentioned above, PTCA is a marker related to indole units linked through the 2-position, which are characterized by extensive π-electron delocalization [38].

To obtain further information on the course of the oxidation reaction of dopamine under the conditions explored, in other experiments the different reaction mixtures were monitored over time by LC-UV-MS analysis and UV-Vis spectroscopy. When dopamine reaction was run with laccase (Method A), a progressive consumption of the catecholamine was observed, reaching a value of *ca.* 80% after 4 h and of 100% after 24 h. In the initial stage of the reactions (2 h), dopamine consumption was coupled to the formation of a main product eluted at *ca.* 9 min (Fig. S7 in the Supplementary Material) characterized by a pseudomolecular ion peak $[M+H]^+$ at *m/z* 150, likely indicative of dopaminochrome. In agreement with this hypothesis, UV-Vis analysis of the reaction mixture (Fig. S7 in the Supplementary Material) at the same time showed the presence of an absorption band at *ca.* 480 nm, which disappeared after addition of a reducing agent such as sodium dithionite. Notably, after reduction, the compound eluted at *ca.* 9 min also disappeared to give rise to a new compound eluted at *ca.* 4 min (Fig. S7 in the Supplementary Material) with a pseudomolecular ion peak $[M+H]^+$ at *m/z* 152. A gradual decay of dopaminochrome was observed over time, as also evident by the UV-Vis analysis (Fig. S7 in the Supplementary Material), and after 24 h no chromatographable compounds were detected anymore. The UV-Vis spectrum of the reaction mixture after 24 h was characterized by the presence of two broad absorption bands at *ca.* 310 and 360 nm, resembling those exhibited by the PDA-coating (Fig. S2 in the Supplementary Material). Overall, these results are in line with those from chemical degradation analysis, highlighting a significant tendency of dopamine to undergo oxidative cyclization, although no traces of DHI or related compounds were found to be present in the oxidation mixture.

As expected, a faster oxidation in the first stages of the reaction was observed under the same conditions but replacing laccase with sodium periodate (Method C). Indeed, a 50% dopamine consumption was observed after 1 h; after that, the catecholamine decayed more slowly, reaching an 81% consumption after 24 h. The initial faster oxidation of dopamine led to the formation of dopaminochrome to a higher (1.8-fold) extent with respect to what was observed with laccase. However, a more complex pattern of unidentified products was present, likely interfering to some extent with PDA-coating formation (cf. Figs. 2 and 3). On the other hand, a very slow consumption of dopamine was observed over the first hours of autoxidation at pH 8.5 (43% after 4 h), with apparently no accumulation of dopaminochrome, which was detected only in trace at a reaction time of 1 h. These data could be likely interpreted based on the higher proneness of dopaminochrome to isomerize to DHI at alkaline pH. The lack of detection of DHI and related species, even in this case, could be attributed to their well-known marked oxidability and, hence, instability at alkaline pHs. This hypothesis would be in agreement with the higher PTCA/PDCA ratio determined in the chemical degradation mixture of both PDA and PDA-coatings produced by autoxidation, compared with that measured for the samples obtained at pH 5.5.

3.3. Laccase-mediated film deposition by sequential dopamine addition over time

Taking into account that in the laccase-mediated oxidation mixture run under optimal conditions (Method A) dopamine consumption was almost complete after 4 h (see above), in a final set of experiments the possibility to make the most of the enzyme catalytic activity and enhance film deposition was investigated. To this aim, the extent of PDA-coating on glass slides by sequential addition of dopamine over time was evaluated. In particular, the reaction was run under the conditions of Method A, but at two different dopamine concentrations (1.25 and 2.5 mM), and after 6 h an equal amount of dopamine with respect to the initial concentration was added to the ongoing reaction. Fig. 6 shows the absorbance value at 400 nm of glasses coated using the different dopamine concentrations, that is 1.25 plus 1.25 mM and 2.5 plus 2.5 mM, and, for comparison purposes, 2.5 and 5.0 mM of dopamine added in one portion. No advantages were apparent when comparing the extent of coating achieved with consecutive additions of 1.25 + 1.25 mM dopamine with that observed with a single addition of 2.5 mM dopamine. However, when a sequential addition of 2.5 + 2.5 mM of dopamine was performed, a significant increase in coating deposition was observed compared to the single addition of 2.5 or 5 mM dopamine. These results suggest that when dopamine is added in a single portion, the reaction occurs faster, altering the delicate balance between coating formation and PDA precipitation, as discussed above. Similar results were previously reported when the PDA film growth mechanisms were studied in the presence of sodium periodate, although in that case an initially higher dopamine concentration (10 mM) was adopted and a second addition of both dopamine and oxidant was needed to enhance film deposition [11]. On the contrary, in the present work, the marked stability of laccase allowed for a sustainable sequential film deposition process, requiring lower dopamine concentration.

4. Conclusions

This work introduces a novel, systematically optimized laccase-assisted approach for PDA film deposition on glass and quartz substrates. It also provides a detailed characterization of the chemical structure and morphological features of the resulting high-quality PDA coatings. Compared to other protocols, laccase was able to induce coating formation under more sustainable conditions, being effective even at low starting concentrations of dopamine and not requiring the use of potentially toxic additives. In addition, the possibility of working at an acidic pH, at which the autoxidation of dopamine is inhibited, may allow for a better control and standardization of the experimental conditions. Finally, the preliminary chemical analysis highlighted some structural differences between the laccase-induced PDA coating and those obtained by autoxidation or periodate-induced dopamine oxidation. All these results provide a significant step forward for the

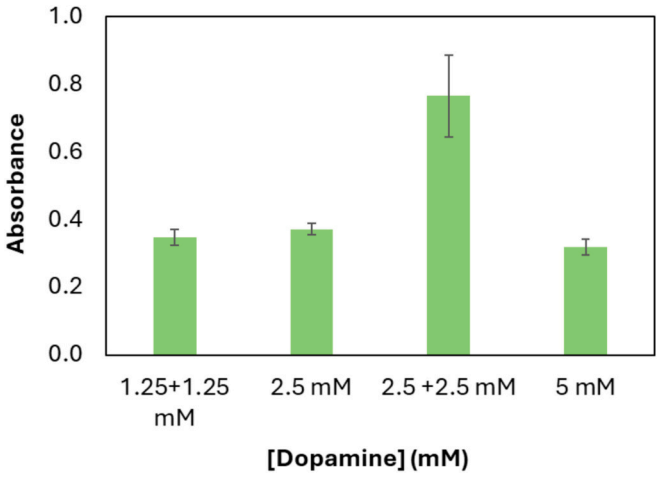

Fig. 6. Absorbance at 400 nm of PDA-coated glasses prepared using different dopamine concentrations, that is 1.25 (t = 0 h) plus 1.25 (t = 6 h) mM or 2.5 (t = 0 h) plus 2.5 (t = 6 h) mM. For comparison, absorbance at 400 nm of the PDA-coated glasses using 2.5 and 5.0 mM of dopamine added in one portion is shown. All reactions were run under Method A conditions. Reported are the mean ± SD values from three individual experiments.

implementation of organic coatings with specific functional characteristics by appropriately selecting the oxidative agent.

CRediT authorship contribution statement

Flávia F. Magalhães: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Maria Laura Alfieri:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Konstantin Romanyuk:** Resources, Methodology, Data curation. **Andrei Kholkin:** Resources, Methodology, Data curation. **Mara G. Freire:** Writing – review & editing, Validation, Supervision, Resources, Investigation, Funding acquisition, Conceptualization. **Alessandra Napolitano:** Writing – review & editing, Validation. **Ana Paula M. Tavares:** Writing – review & editing, Validation, Supervision, Investigation, Conceptualization. **Lucia Panzella:** Writing – review & editing, Validation, Supervision, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.porgcoat.2025.109399.

Data availability

Data will be made available on request.

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