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Synthesis and characterization of melanin in DMSO

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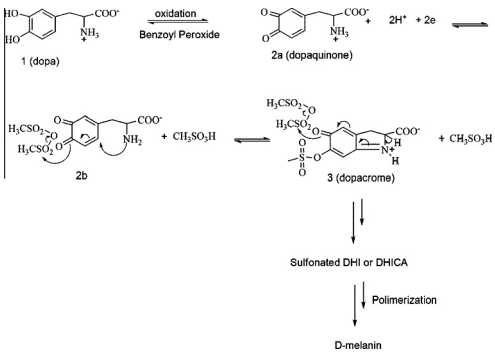
HIGHLIGHTS

- Study on the structural characteristics of melanin synthesized in water and DMSO.
- Sulphonate groups (—SO_2CH_3) in the monomeric structure of D-melanin.
- Sulphonate groups render the compound soluble in DMSO.
- The synthetic method has been widely cited in literature.
- The structure was not understood until this structural study.

GRAPHICAL ABSTRACT



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ABSTRACT

Recently soluble melanin derivatives have been obtained by a synthetic procedure carried out in DMSO (D-melanin). In this work a comparative study of the structural characteristics of synthetic melanin derivatives obtained by oxidation of L-DOPA in H_2O and DMSO are presented. To this end, Fourier-transform infrared spectroscopy as well as proton and carbon nuclear magnetic resonance techniques has been employed. In addition, aging effects have been investigated for D-melanin. The results suggest that sulfonate groups (–SO_2CH_3) from the oxidation of DMSO, are incorporated into melanin, which confers protection to the phenolic hydroxyl group present in its structure. The solubility of D-melanin in DMSO is attributed to the presence of these groups. When D-melanin is left in air for long time periods, the sulfonate groups leave the structure, and an insoluble compound is obtained. NaOH and water have been used, in order to accelerate the release of the sulfonate groups attached to D-melanin, thereby corroborating the proposed structure and the synthesis mechanism.

1. Introduction

Melanins represent an important class of natural pigments due to their biological properties regarding photoprotection as well as technological applications. These compounds occur both in plants and animals. [1] Dark coloration, high insolubility, resistance to hydrolysis, lack of molecular regularity, and paramagnetism are some of the typical features of these biopolymers [2–4].

In particular, melanins present interesting optoelectronic properties such as high optical absorption in the UV–Vis range, good electron conductivity, appreciable ionic conductivity, and memory switching, which make them promising active components in organic electronic devices of low environmental impact [5–7].

Due the difficulty in extracting melanin from its natural sources, different synthetic routes were proposed [3,8–10]. Traditionally melanin is synthesized using the: (i) oxidation of tyrosine

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or L-3,4-dihydroxyphenylalanine (L-DOPA) via enzymatic reactions, and (ii) auto-oxidation of L-DOPA in alkaline aqueous solution. In both cases water is used as solvent. Theses syntheses occurs in an uncontrolled manner, thereby culminating in a polymeric material with a high degree of chemical heterogeneity, and the resulting material (H-melanin) is as expected insoluble in water and common solvents [1].

Owing to the lack of molecular regularity, it is believed that the melanin structure is mainly composed of macromolecules of several redox forms of 5,6 dihydroxyindole quinone (DHI or HQ) and 5,6 dihydroxyindole-2 carboxylic acid (DHICA). The oxidized forms of DHI and DHICA, namely dihydroxyindole (HQ), semiquinone (SQ), indole quinone (IQ), and quinine imine (QI), are also expected [11], but the details of how these units connect is still under debate. Many different structures were proposed [7]. Nevertheless, the best-accepted hypotheses describes melanin as graphene-like planar structures of a few nanometer in diameter. These small planar structures made of DHI, DHICA as well as their oxidized forms, are stacked into two or three sheets [12].

The low solubility or insolubility of this compound in different organic solvents makes its characterization a difficult task, but more importantly the production of good-quality thin films a challenge [13]. Notice that in principle melanin is biocompatible, which makes it ideal for bioelectronics [13]. Melanin thin films have been obtained by using alkaline aqueous solutions, where melanin is soluble [14,15]. The solubility of melanin in basic solutions is believed to come from the deprotonation of the various acid/base moieties found in melanin, including carboxylic acid and phenolic groups [5,6,14]. Concentrated hydroxide solutions were used in these works and as a consequence though homogeneous films were deposited, they were contaminated with sodium hydroxide.

Recently, soluble melanin derivatives have been prepared by means of a synthetic procedure based on the oxidation of L-DOPA in DMSO with benzoyl peroxide (D-melanin) [2,16]. The resulting material, D-melanin was used to prepare homogeneous films with good adhesion to the substrate [2]. However, no satisfactory answer was given on why this material is soluble in DMSO, or in other words what were the differences in D-melanin structure that conferred this property.

In the present work the structure of D-melanin is investigated, as well as the reaction mechanism involved in the synthesis. For this purpose spectroscopic techniques such as Fourier-transform infrared spectroscopy (FTIR), carbon cross-polarization "magic angle spinning" (^{13}C CP/MAS), and proton nuclear magnetic resonance (^{1}H NMR) were used. The results indicate the presence of sulfonate groups in the freshly-prepared D-melanin, which confers solubility to the compound. A reaction mechanism is proposed that explains our experimental findings. DMSO is oxidized and as a consequence phenolic oxygen present in melanin are sulfonated. The proposed mechanism was tested using a simple chemical reaction. Freshly made D-melanin was made insoluble after a nucleophilic substitution of its sulfonate groups.

2. Experimental

2.1. General

All the commercially available chemicals were purchased from Acros or Sigma–Aldrich and were used without further purification.

2.2. Techniques

FTIR measurements were conducted on a Bruker Vertex 70 Fourier Transform spectrometer in the region between 400 and 4000 cm^{-1}, in the ATR mode (Attenuated Total Reflectance).

^{13}C CP/MAS analyses were performed on a 9.4-T Varian INOVA 400 spectrometer, operating at frequencies of 100.5 MHz and 400 MHz for ^{13}C and ^{1}H, respectively. To this end, a VARIAN 5-mm "magic angle spinning" – MAS – probe (Jackbosen design) was used. The ^{13}C CP/MAS spectra of the solids were obtained by means of the cross-polarization technique (Cross-Polarization Magic Angle Spinning – CPMAS) with contact time of 1 ms, repetition time of 5 s and MAS rotation frequency of 5 kHz. Total Suppression of Spinning Sidebands (TOSSs) was used.

^{1}H NMR spectra were acquired on a 400-MHz Bruker DRX 400 spectrometer. For these measurements, D-melanin was dissolved in deuterated dimethylsulfoxide (DMSO-d_6). The ^{1}H NMR spectra were interpreted with the aid of the simulation software Chem Draw Ultra [17].

2.3. Synthesis

2.3.1. Synthesis of H-melanin

H-melanin was synthesized by L-DOPA auto-oxidation [18]. L-3,4-dihydroxyphenylalanine, L-DOPA (Sigma–Aldrich), was mixed twice-distilled deionized water. The pH was decreased using NaOH (2M) and the solution was air bubbled for 27 days by means of an air pump. In the end of the reaction, H-melanin was filtered using a dialysis membrane of 5000 Daltons. After separation, H-melanin was dried by heating at 60 °C for 48 h. The final product is a black powder.

2.3.2. Synthesis and processing of D-melanin

For the synthesis, 3 g L-DOPA (Sigma–Aldrich) were mixed with 3.7 g benzoyl peroxide in 400 mL dimethyl sulfoxide. The reaction mixture was kept under agitation for 21 days, at room temperature. The solution was then heated at 140 °C, for evaporation of the solvent. The concentrated solution of melanin in DMSO was then mixed with acetonitrile (Sigma–Aldrich). D-melanin precipitates in acetonitrile, so a sequence of centrifugation steps followed by extraction of the precipitate provided a purified powder of D-melanin. D-melanin degrades with time, so samples that remained on the shelf for over 2 years and became insoluble were designated D-melanin-aged. To test the nature of the aging process and the loss of solubility, a NaOH (2M) aqueous solution (0.5 mL) was added to 20 mg of D-melanin in DMSO (4 mL). This mixture was left under stirring for 30 min. After this period, a black powder precipitated, which we shall name as D-melanin-degraded.

3. Results and discussion

3.1. D-melanin structure evaluation

As already mentioned, freshly prepared D-melanin is soluble in DMSO. However, neither its solubility in different solvents nor its stability was reported. Therefore, solubility tests were conducted in different solvents, namely methanol, tetrahydrofuran, acetone, ethyl acetate, chloroform, water, and dimethyl sulfoxide. The solubility tests were conducted for D-melanin, as well as in H-melanin, and D-melanin-aged. The results revealed that H-melanin and D-melanin-aged is insoluble in all these solvents, whereas freshly prepared D-melanin is soluble in DMSO only [2,12]. The fact that D-melanin loses its solubility with time (D-melanin-aged) is noteworthy and suggests that it is composed of unstable chemical moieties.

FTIR, ^{1}H NMR, and ^{13}C CP/MAS were used to analyze the structural differences between H-melanin, D-melanin, and D-melanin-aged. Fig. 1 presents the FTIR spectra for different melanin samples.

As can be seen, there is significant structural differences between all three samples D-melanin, H-melanin, and

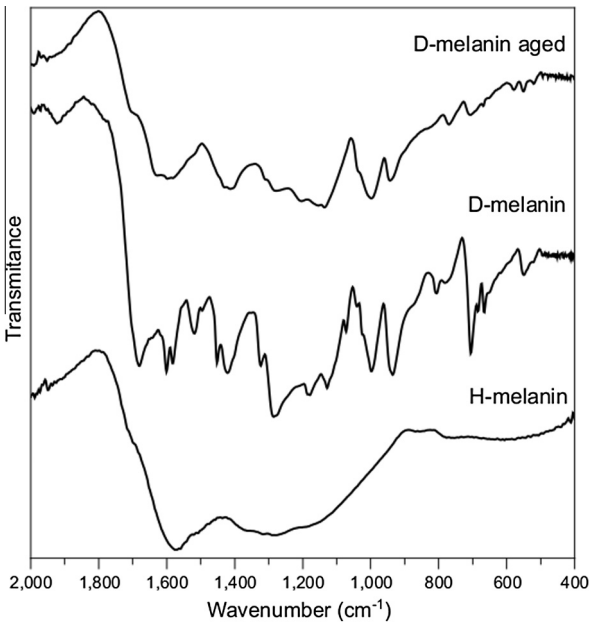


Fig. 1. FTIR spectra of the different synthetic melanins: H-melanin, D-melanin, and D-melanin aged.

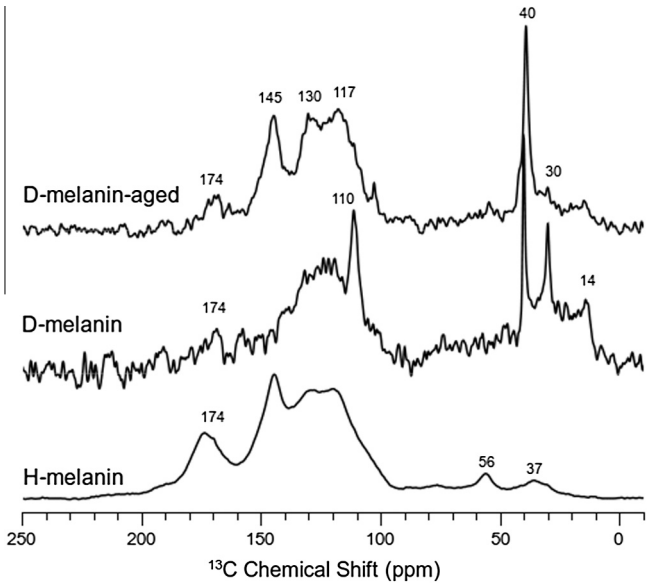


Fig. 2. ^{13}C CP/MAS NMR spectra of the different synthetic melanins: H-melanin, D-melanin, and D-melanin aged.

D-melanin-aged. All spectra show the typical bands observed in melanin. A large band between 3500 and 2400 cm^{-1} is observed, not shown, is associated with —OH and —NH stretching modes of the carboxylic acid (C—O and —COOH), phenolic (C—O/carboxyl OH) and aromatic amino functions presents in the indolic and pyrrolic systems present in DHI and DHICA derivatives. The band at 1685 cm^{-1} is assigned to the C=O stretching mode of carboxylic acid (—COOH). The bands at 1610–1650 cm^{-1} corresponds to aromatic C=C and C=N bending modes and C=O stretching mode from noncarboxylic acid moieties. The C=O stretching mode of ionized carboxylic acid (COO^-) is seen at 1579 cm^{-1} and the region between 1180 and 1295 cm^{-1} is associated with stretching mode bands related to carboxilic acid and phenolic groups [19,20]. However, D-melanin and D-melanin-aged have new bands. Table 1 lists the new vibrational modes detected in D-melanin.

From Table 1, the main differences in the FTIR from H-melanin and D-melanin are related to sulfonate —SO_2X groups in D-melanin (1900–400 cm^{-1}). After storage, see Fig. 1, there is a reduction in the bands attributed to the sulfonate groups (700–600 cm^{-1}: C—S stretching, 830–690 cm^{-1}: S—O stretching, 1420–1330 cm^{-1}; SO_2 asymmetric stretching), which suggests that these groups leave the molecule after some time. These results suggest that solubility in DMSO may come from the —SO_2X groups [19].

Fig. 2 shows ^{13}C CP/MAS spectra for the same group of samples.

The spectra show three broad regions of carbon resonance attributed to aliphatic, aromatic and carbonyl functions. The bands at 37 and 56 ppm results from uncyclized aliphatic chains in the polymer or from unreacted L-dopa remaining in the solution [3]. The signal found in the 90–135 ppm region is related to indole and pyrrole structures. The 140–160 ppm region is assigned to aromatic carbons bearing oxygen while the signal at 174 ppm is related to carbonyl groups. These results are in good agreement with the literature [3].

Again, there are significant modifications in the spectrum of D-melanin as compared to H-melanin. The intensity of the signal ascribed to C=O (174 ppm) diminishes, and the signal assigned to phenol hydroxyl C—OH (145 ppm) disappears in the case of D-melanin [3,21,22]. New signals are also verified at 40 and 30 ppm in D-melanin, which can be attributed to sulfonate groups (—SO_2CH_3) bound to phenolic oxygen atoms or to the protection of the indole nitrogen by methyl groups (—N—CH_3) generated during the synthesis, as will be further discussed [19]. In D-melanim, there is no signal in the region around 56 ppm. On the other hand, the signal at 110 ppm assigned to pyrrole carbons is clearly observed in D-melanin, suggesting L-DOPA cyclization and/or a possible polymerization spot between two DHI or DHICA units [23].

Aging is also observed to affect the ^{13}C CP/MAS spectra. D-melanin-aged has the 145 ppm signal found in H-melanin which indicates that the sulfonate groups leave the compound and are substituted by hydrogen. In good agreement with the FTIR data discussed above.

Fig. 3 shows the ^1H NMR spectrum of D-melanin in deuterated DMSO. This analysis was only possible in D-melanin since the other samples are insoluble in DMSO. The disordered nature of D-melanin does not allow a quantitative analysis of the signal or the evaluation of J_{HH} spin coupling.

Chem Draw Ultra [17] was used for the assignment of the bands observed: the region lying between 6.5 and 7.2 ppm is related to protons belonging to the double bond of the indole ring, the region between 7.0 and 8.2 ppm corresponds to two aromatic ring protons, and the broad signal around 9–10 ppm is ascribed to protons bound to nitrogen. The signals appearing from 2.5 to 3.67 ppm cannot be attributed to any basic melanin structure (DHI and DHICA), so they are probably associated with structural alterations taking place during the synthetic procedure [17,24]. Notice that the protons found in acid function should appear at approximately 12.0 ppm, not detected.

The presence of sulfonate groups had already been revealed by FTIR and ^{13}C CP/MAS and is corroborated by the signal at 3.67 ppm,

Table 1
New FTIR absorption bands found in D-melanin.

IR band (cm^{-1})	Assignment	
1420–1330	—SO_2	Asym stretching
1235–1145	—SO_2	Sym stretching
1020–850	—SO	Asym stretching
830–690	—SO	Sym stretching
700–600	—C—S	Stretching

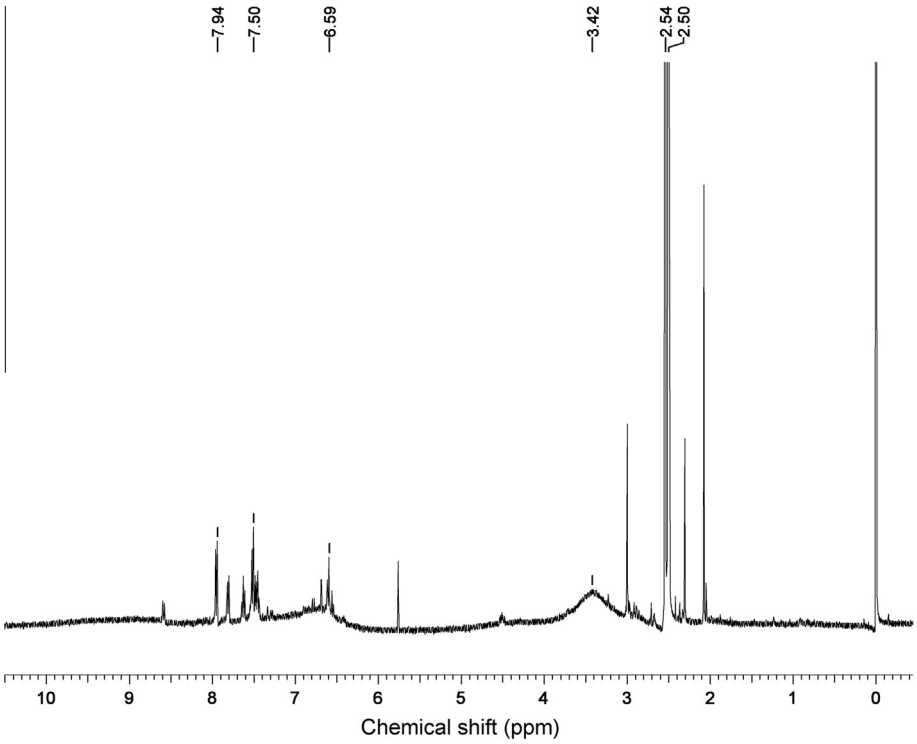


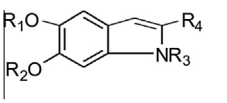
Fig. 3. ^{1}H NMR spectrum of D-melanin freshly synthesized in DMSO d_6.

assigned to the protons of the $-SO_2CH_3$ group. This broad singlet signal, has its shape probably because of strong relaxation process undergone by the hydrogen atoms adjacent to the sulfur atom, which is a clear indication of O-sulfonation. Methylation of the nitrogen atom would also be evident in this same spectral region, but these groups would not be enough to confer solubility to the compound in DMSO [24]. The signals at 2.5 ppm are associated with DMSO, which comes from the solvent deuterated DMSO or sulfonate groups bound to the pyrrole nitrogen; i.e., relative to the occurrence of N-sulfonation [17,25].

The signals at 30 and 3.6 ppm observed in the ^{13}C CP/MAS and ^{1}H NMR spectra, respectively, suggest nitrogen methylation [24]. Nevertheless, the ^{1}H NMR data are inconclusive, as discussed before. Methylation of the nitrogen atom may be associated with the oxidation of dimethylsulfone in the reaction medium [26] or to its thermal decomposition during the process of extraction [27]. ^{15}N NMR analysis would be necessary for total elucidation. This analysis is underway.

In Fig. 4 the possible structures that constitute D-melanin are presented based on the experimental observations just discussed.

The presence of protecting groups at R1 and R2 and/or R3 would account for the solubility of the compounds in DMSO, in the same way that acetylated and methylated melanins are soluble in organic solvents [23,24].



R1 = SO_2CH_3 or H
R2 = SO_2CH_3 or H
R3= SO_2CH_3 , CH_3 or H
R4 = COOH or H

Fig. 4. Basic structures that compose D-melanin.

3.2. Evaluation of the mechanism involved in the synthesis of D-melanin.

Despite the interesting properties of D-melanin, the basic aspects concerning its synthesis are little known. In this context, the FTIR spectra of a mixture consisting of DMSO and the peroxide have been analyzed, aiming to assess the role of benzoyl peroxide in the synthesis of melanin. Fig. 5 shows the FTIR spectrum of DMSO, Benzoyl peroxide and DMSO/benzoyl peroxide mixture (2:3 mol/mol) left for 30 min after mixing.

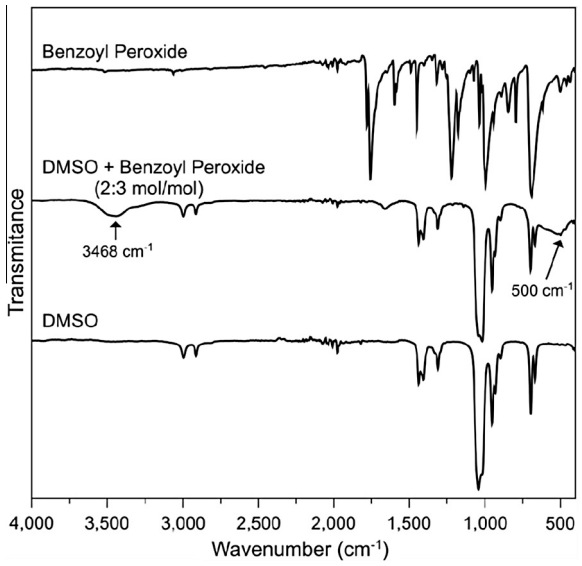
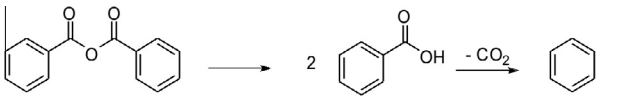
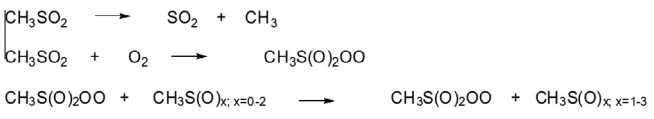


Fig. 5. FTIR spectra of DMSO, benzoyl peroxide, and the solution of DMSO + benzoyl peroxide, 30 min after mixture.

The DMSO principal bands are the C—H stretching (2900 cm^{-1}), C—C stretching (1440–1413 cm^{-1}) and S—O stretching bands at 1050 cm^{-1} and 954 cm^{-1}. Benzoyl peroxide spectrum shows two bands in 1780 and 1753 cm^{-1} from C=O stretching, bands at 1600 cm^{-1} related to vibrations of phenyl ring. The bands at 1224 cm^{-1} is relative to C—O stretching and that at 1000 cm^{-1} is very likely related to O—O stretching vibrations. For the DMSO/benzoyl peroxide mixture, the C—O stretching bands of benzoyl peroxide are not observed. There are new bands at 500 cm^{-1}, relative to the SO_2 groups, suggesting the formation of dimethylsulfone or other sulfonated derivatives. This is expected from the mechanism proposed which involve DMSO oxidation. The band at 3400 cm^{-1} refers to the absorption of water during the oxidation of DMSO. In fact if one follows the reaction during 40 min one observes an increase in the intensities of the bands highlighted in Fig. 5 with time (data not shown) [28]. In this mechanism, DMSO solvates the benzoyl peroxide in the reaction medium, thereby weakening the O—O bond and enhancing its reactivity. After cleavage of the benzoyl peroxide, one oxygen atom is transferred to the DMSO molecule, thus producing dimethylsulfone ($DMSO_2$) and benzoic anhydride [28]. The absence of bands attributed to carbonyl groups (1750 cm^{-1} and 1224 cm^{-1}) and the presence of a weak band assigned to aromatic ring (~1600 cm^{-1}) is a clear indication of the degradation of benzoic anhydride, according to Scheme 1. Besides promoting the oxidation of DMSO, it is also expected that the benzoyl peroxide favor the oxidation of L-DOPA in the reaction medium.

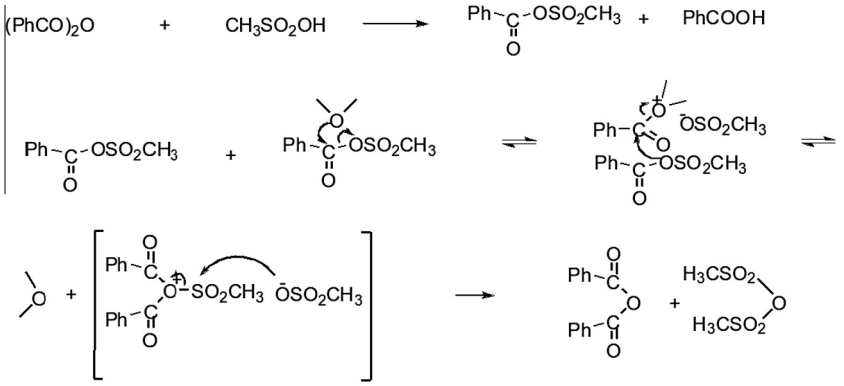


Scheme 1. Mechanism for the degradation of benzoic anhydride.

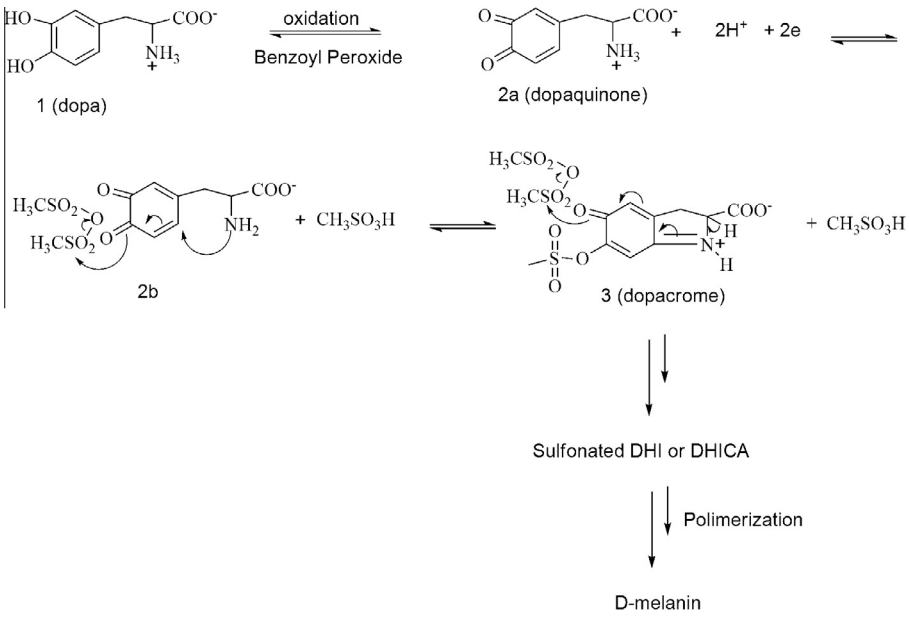


Scheme 2. Mechanism for the oxidation of dimethylsulfone.

The process of DMSO oxidation to dimethylsulfone is well established in the literature, but the processes involved in $DMSO_2$ oxidation and the generation of methane sulfonic acid (CH_3SO_2OH) are not clear. The formation of this compound may be associated



Scheme 3. Mechanism for the formation of sulfonic anhydride.



Scheme 4. Reaction mechanism with the molecules formed during the synthesis of melanin in DMSO.

with the oxidizing medium in a number of ways, such as the degradation of $DMSO_2$ according to the mechanism proposed by Arsene et al. [29] (see Scheme 2).

The initial step in the oxidation and polymerization of melanin in water is the production of a quinone with a free radical intermediate, in this case dopaquinone, according to the mechanism proposed by Mason and Rapper [30,31]. Cyclization of dopaquinone takes place in basic medium, giving rise to cyclodopa, which is then oxidized to dopachrome, in a conversion involving four electrons per molecule. Finally, a series of oxidations occur on dopachrome, producing DHI and DHICA as well as their oxidized forms, thereby initiating the process of melanin polymerization [31].

In the case of D-melanin, as verified in the ^{13}C CP/MAS spectra, sulfonated compounds generated from the oxidation of DMSO participate in the synthesis of D-melanin. These groups protect the phenolic oxygen atoms and/or nitrogen atoms of the DHI or DHICA monomers see Fig. 4. The protection of the phenolic hydroxyl groups can be assigned to the presence of methanesulfonic anhydride in the reaction medium, a result of the reaction between benzoic anhydride and methanesulfonic acid, as represented in Scheme 3 [32]. This compound is known for protecting hydroxyl groups efficiently in alkaline medium, by means of nucleophilic substitution reactions [33,34].

Another possibility is that the hydroxyl reacts with methanesulfonic acid, eliminating a water molecule. According to Teasdale et al. [35], the reaction between sulfonic acid and alcohol follows a first-order kinetic pathway, which implies that the reaction occurs almost exclusively between the protonated alcohol and the sulfonate anion ionic pairs. When water is added to this system, there is a drastic reduction in the formation of sulfonated ester, due to competition between protonation by water and the alcohol attack. Consequently, the ratio of the second-order reaction is increased, and there is a tenfold increase in the formation rate of sulfonate ester hydrolysis as compared to the previous formation rate of sulfonate ester production [35]. Thus, anhydrous conditions tend to favor the generation of sulfonated esters on the phenolic hydroxyls.

The proposed mechanism assumes the regeneration of benzoic anhydride, which later undergoes hydrolysis. The existence of methanesulfonic anhydride is evident from the —SO_2 asymmetric stretching bands at 500 cm^{-1} in the FTIR spectrum, see Fig. 5 [19].

Scheme 4 depicts the reaction mechanism proposed on the basis of the previous discussion. The possible structural derivatives obtained during the process of melanin synthesis in DMSO are presented, including the cyclization of dopaquinone to dopachrome.

The proposed reaction mechanism is similar to the one proposed for the synthesis of H-melanin in basic medium. The difference lies on the action of methanesulfonic anhydride at the time of dopachrome formation.

The incorporation of sulfonate groups in the oxygen atoms of the aromatic hydroxyls or in the nitrogen atom of the pyrrole ring after cyclization are expected. These reactions are known as O-sulfonation and N-sulfonation. The reaction with hydroxyl is more probable as compared to with amines [33]. Indeed, the absence of FTIR absorption bands in the region of 1360–1315 cm^{-1}, relative to N—S bond stretching [19], suggest that the incorporation of sulfonate groups in the nitrogen atom of the pyrrole ring did not happen.

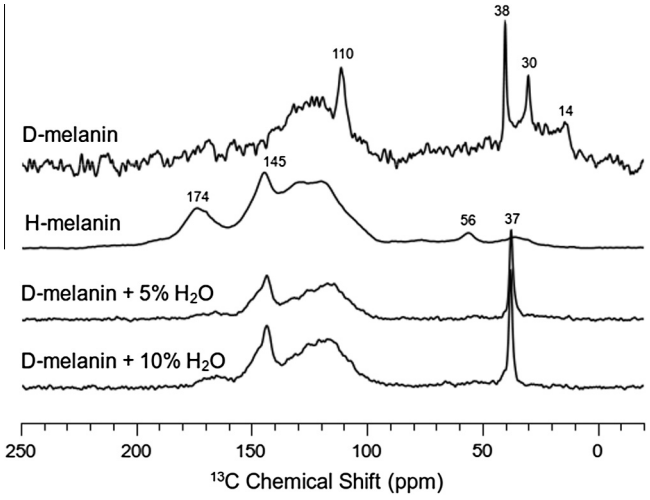


Fig. 6. ^{13}C CP/MAS NMR spectra of the different synthetic melanins: D-melanin, H-melanin, D-melanin + 5% H_2O and D-melanin + 10% H_2O.

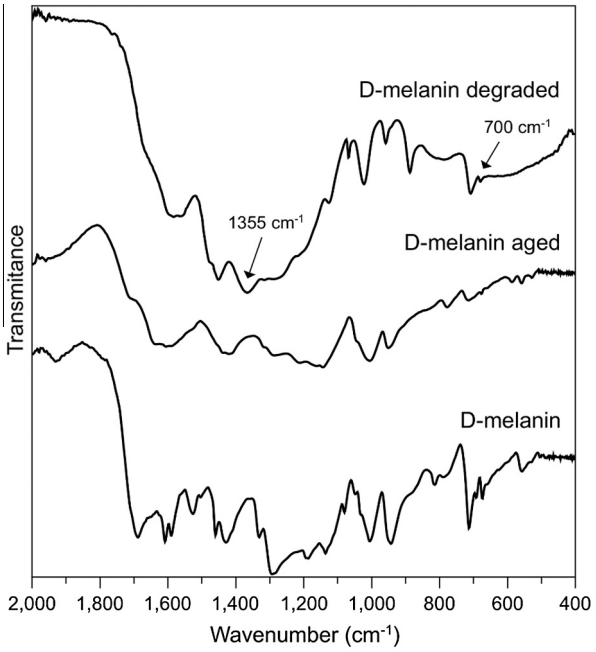
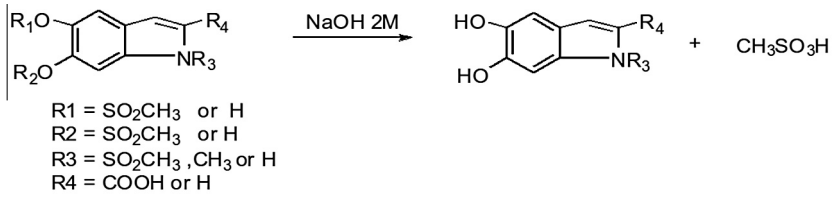


Fig. 7. FTIR spectra of D-melanin, D-melanin aged, and D-melanin degraded.



Scheme 5. Degradation mechanism of D-melanin in NaOH solutions.

From what was previously discussed water can be used to unveil the synthesis steps of D-melanin. For that purpose water was added during synthesis. Fig. 6 shows the ^{13}C CP/MAS NMR spectra of D-melanin, H-melanin, and melanin synthesized in DMSO with the addition of 5% and 10% water to the total volume of the solution. The spectra are similar to the ones in Fig. 2. As can be seen the addition of water makes the ^{13}C CP/MAS NMR spectra of melanin synthesized in DMSO similar to H-melanin. This result is in good agreement with the proposed mechanism, since it is expected that the presence of H_2O hydrolyzes the products from DMSO oxidation in the reaction medium, thereby preventing the sulfonation of DHI and DHICA.

In order to confirm the O-sulfonation proposed for D-melanin (Fig. 4) and its instability as observed in D-melanin-aged, a reaction of D-melanin with an aqueous solution of NaOH was conducted at room temperature [36]. It is known that the addition of NaOH to solutions containing compounds such as the mesylate group (—O—SO_2CH_3) in D-melanin promotes their removal, as described in Scheme 5 [33,34,37]. On the other hand, NaOH is not effective on N-sulfonamides and/or the demethylation of nitrogen, since these groups are more stable [38,39].

It was observed that after 10 min of reaction a precipitate is formed. The precipitate was extracted, named D-melanin-degraded and the resulting solid was analyzed by FTIR. This observation supports the hypothesis that the sulfonate groups confer solubility to D-melanin in DMSO.

Fig. 7 shows the FTIR spectra for D-melanin, D-melanin-aged, and D-melanin-degraded. As can be seen the spectra are similar to ones described in Fig. 1. However in the case of D-melanin-degraded one can observe a band at 1350 cm^{-1} assigned to the sulfonic acid generated from the removal of the sulfonate groups of D-melanin, with decrease of band at 700 cm^{-1} referent to C—S stretching.

4. Conclusions

In this work we have studied the synthesis of melanin in DMSO. For that purpose we have analyzed the structure of melanin prepared under different synthetic conditions as well as different processing.

Our results show that melanin synthesized in DMSO (D-melanin) has sulfonate groups (—SO_2CH_3) that substitute preferentially H in phenolic oxygen. We propose that the sulfonate derivatives are formed during DMSO oxidation in the reaction medium

The sulfonate groups are responsible for the solubility of D-melanin in DMSO. Moreover, these groups are found to be unstable. They leave the compound with storage time. Or they can be removed with the use of NaOH solutions. In both cases the removal of the sulfonate groups is found to leave D-melanin insoluble.

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