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Titanium based complexes with melanin precursors as a tool for directing melanogenic pathways

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Abstract: Bioinspired melanin based hybrid materials hold huge promise for developing multifunctional systems for a considerable set of applications. Yet, control of melanogenic pathway is a big challenge to allow rational design of nanostructured systems with tuned structures and improved properties. This study proves the ability of titanium ions interactions with melanin precursors in directing melanogenic pathway. To this purpose complementary spectroscopic evidences were collected to reveal that in the presence of a TiO_2 -sol, amino-acid complex of Ti(IV) ions and DOPA actually inhibits its cyclization, during oxidative process, thus leading to DOPA-based polyphenols, stable even in oxidative environment, rather than eumelanin. This hugely impacts on the biological properties of the final hybrid systems which, discloses relevant and durable antioxidant behavior but poor antimicrobial activity differently from DHICA-based hybrid nanostructures. Overall this study, discloses the high potential of ceramic templated approach in combination with the selection of melanin precursor in achieving a fine tuning of physico-chemical as well as bioactivity of melanin- TiO_2 nanostructures, opening new scenarios towards the design of cutting-edge biomaterials with tailored biological properties.

Keywords: antioxidant properties; DOPA; Eurasia 2018; melanin based hybrid materials; Ti(IV)-DOPA complexes.

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Introduction

Melanins are a class of ubiquitous bioavailable pigments widely distributed in microorganisms, plants and animals, where they play significant physiological roles [1] ranging from metal ion chelation, photoprotection, free radical quenching, proteins binding as well as antimicrobial and inflammatory functions [2]. Their intrinsic multifunctional behavior and intriguing physico-chemical properties have boosted the interest of chemists and materials scientists to design highly performing systems for a broad range of technological and biomedical applications [3–5]. However, despite the flourishing number of studies on melanins, to date there is no shared explanation of their manifold available colors, sizes and functions. Actually, their structure is not completely understood and its influence on their properties and behavior is still to be unraveled [6, 7].

Most of biologically available melanins are produced via a multistep synthesis pathway, starting from the amino acid L-tyrosine as common precursor, which undergoes tyrosinase-catalyzed oxidation to DOPA (o-dihydroxyphenylalanine) and dopachrome. Then, the cyclization further mediates to form 5,6-dihydroxyindole (DHI) or 5,6-dihydroxyindole-2-carboxylic acid (DHICA) [8, 9]. Oxidative polymerization of these indoles, finally gives rise to the black-brown variety of eumelanin biopolymers. This multistep process occurs in melanosomes, specialized membrane-encased organelles, where melanin is synthesized, structured through interactions with structural proteins and stored [10].

Actually, it has been widely recognized that formation environment hugely impacts on growth kinetics and architecture of melanins, both *in vitro* and *in vivo*, and ultimately affects their functions [11, 12]. Notably, metal-binding ability of melanogenic precursors can give rise to stable complexes with available metals in physiologic environment, thus influencing the course of melanins formation and ultimately determining their features [13, 14]. Particularly, some metal ions, such as copper, proved to enhance the rearrangement of dopachrome to form DHICA, thus determining its higher amount than DHI in natural melanins [13, 15].

In light of these advancements, many studies have been trying to assess the function of metals in melanins formation pathway as well as in defining both composition and structure of obtained biopolymers [16]. To this purpose, studies on natural melanins cannot provide reliable information, due to the difficulty to keep native physicochemical properties during purification and extraction processes [17]. Synthetic systems, prepared through oxidation of melanin precursors under biomimetic/bioinspired conditions, can efficiently act as a model of the natural pigments in that they reproduce similar physical-chemical properties, ensuring at the same time reproducible and measurable formation conditions [18], thus contributing to shed light on these complex polymeric materials and their huge functions in natural systems [14].

In this context, ceramic nanostructured phases proved their ability to catalyze and tune melanin structure during its formation, starting from melanogenic precursors, thus performing the same functions as melanosomes. Actually, bioinspired ceramic templated approach holds a unique opportunity to enhance melanin bio-functional properties while at the same time providing significant contribution to discern the influence of synthesis conditions on melanin structure and functions [4, 5, 9, 19, 20].

In this route, pioneering studies disclosed the role of DHICA-Ti(IV) ions ligand to metal charge transfer complex (LMCTC) in boosting DHICA oxidative polymerization to melanin, leading to DHICA-Eumelanin-TiO₂ nanostructures with unique antimicrobial activity even under visible light [21].

These melanin-based nanosystems (NSs) showed a peculiar mechanism of action, whose strong interactions with bacterial membranes enhanced the action of reactive oxygen species (ROS) produced by eumelanin degradation itself, also concurring with the final biocide activity [4, 5, 20].

This study aims at elucidating the role of Ti(IV) interaction with melanogenic precursors in modulating its reactivity in the melanogenic pathway (Fig. 1) and consequently biological performance of produced biopolymers. To this purpose, ceramic templated approach was implemented using a TiO₂-sol and L-3,4 dihydroxyphenylalanine (DOPA) as melanin precursor. DOPA is involved in the initial steps of melanogenic pathway, before cyclization reactions and bears different functional groups able to coordinate Ti(IV) ions. Complementary analysis methodologies, UV-Vis and Electron Paramagnetic Resonance (EPR) spectroscopies, were employed to investigate system features both during synthesis procedure and as a final product, which

Fig. 1: Simplified scheme of synthesis path for melanin production.

was further characterized through Transmission Electron Microscopy (TEM) and BET porosimetry. Results of physico-chemical characterization revealed that amino-acid complex of DOPA and Ti(IV) ions actually inhibits its cyclization, during oxidative process, thus leading to polyphenolic oligomers rather than melanin. This markedly influenced antimicrobial and antioxidant behavior of the final hybrid nanostructures. From a fundamental point of view, this study provides significant contribution to disclose the role of metal ions during melanogenic process.

On an applied research level it will have impact on the design of melanin-based hybrid systems with optimized performance in myriad applications from electronics, to electrocatalysis, biosensors and biomedical systems.

Experimental section

Synthesis of DOPA-based/TiO₂ nanostructures

L-3,4-diidroxphenylalanine (DOPA) polymerization in the presence of TiO₂-sol was realized by a bioinspired approach following a hydrothermal synthetic protocol as defined by our research group to obtain antimicrobial DHICA-melanin/TiO₂ nanostructures [4, 5]. Different ratios of the two precursors, titanium isopropoxide (TTiP) and DOPA (both obtained from Sigma-Aldrich) were used to synthesize the hybrid organic/inorganic nanostructures. In details, a typical inorganic precursor solution was obtained by adding dropwise 6 mL of a 1.69 M solution (Sol-1) of TTiP in isopropanol (from Sigma-Aldrich) to 31.3 mL of water solution at pH 1.5 (Sol-2) achieved by means of acetic acid (from Sigma-Aldrich). A white precipitate was obtained after Sol-1 addition leading to a yellowish suspension upon stirring at room temperature for 2 days, thus indicating

resuspension of the precipitate and the formation of a colloidal suspension composed by small TiO_2 nanoparticles. Subsequently, a specific amount of DOPA (20 mg for 1.193 mL of TTiP) was added to the TiO_2 colloidal suspension just before the dropwise addition of triethylamine (TEA), also obtained from Sigma-Aldrich, to neutralize until $\text{pH} = 7$. In order to well investigate the behavior of DOPA in the presence of Ti(IV) , different weight ratios between the organic and inorganic precursor were used. An orange suspension was obtained and then sealed within a Teflon recipient (the liquid volume corresponding to 75 % of the whole), placed into a circulating oven and kept at 120°C overnight. Hybrid DOPA-based/ TiO_2 nanostructures were recuperated by centrifugation and repeated washing (3 times) with distilled water. The obtained samples are indicated as $\text{TiO}_2\text{DOPA_ti}$. Then, aliquots of the $\text{TiO}_2\text{DOPA_ti}$ suspensions were also submitted to oxidation by exposure to an oxidizing atmosphere (i.e. oxygen atmosphere and ammonia vapors). Specifically, each suspension was incubated for 4 h in the oxygen/ammonia atmosphere at controlled temperature ($25\text{--}40^\circ\text{C}$). The ammonia vapors were produced by equilibration of the atmosphere with ammonia solution (28 %–7 % NH_3 in H_2O) in a sealed camera at 1 atm pressure.

The samples obtained after this procedure are indicated as $\text{TiO}_2\text{DOPA_polym}$. Finally, bare DOPA-eumelanin was also prepared as a reference by following a synthetic procedure described in literature [22]. Indeed, the biological and physico-chemical investigations were principally focused on the samples prepared with the higher DOPA amount, corresponding to the analogous obtained by using DHICA as organic monomer which have been demonstrated the best biological activities.

Antimicrobial assays

The antimicrobial activities of TiO_2 , $\text{TiO}_2\text{DOPA_ti}$ and $\text{TiO}_2\text{DOPA_polym}$ samples were evaluated against *Escherichia coli* DH5 α and *Staphylococcus aureus* ATCC 6538. A single colony of each strain was resuspended in 5 mL of Luria-Bertani (LB) broth and incubated overnight at 37°C . When the culture reached an OD_{600} of 1 unit, it was diluted 1:100 in 20 mM phosphate buffer at pH 7.0. NSs suspensions were sonicated on ice with a tip-sonicator at 50 % amplitude for 10 min (alternating 30 s on/off) before to prepare the mixture with bacteria. In dose-response curves, the samples were prepared by adding 1/25 of the volume of bacterial cells and NSs suspensions were used at different concentrations (from 0 to 400 $\mu\text{g/mL}$), 500 μL final volume was reached with 20 mM phosphate buffer at pH 7.0. Negative control was represented by no treated cells. Samples were incubated at 37°C for 10 min or 4 h, then two dilutions (1:100 and 1:1000) of all the samples were placed on solid medium LB agar and incubated overnight at 37°C . The following day, the surviving cells were estimated by colony counting on each plate and compared with the controls. Standard deviations were less than 5 % for each experiment (which was performed at least in triplicate). The same assay was performed on *Escherichia coli* DH5 α cells incubated with NSs suspensions stored for different time (0, 2, 5, 15, 21 days) at a fixed concentration of 200 $\mu\text{g/mL}$ [23].

ATP leakage measurements

Escherichia coli DH5 α were grown to mid-logarithmic phase in Muller Hinton Broth (MHB) at 37°C . The bacterial suspension (1 mL) was incubated with TiO_2 , $\text{TiO}_2\text{DOPA_ti}$ and $\text{TiO}_2\text{DOPA_polym}$ (200 $\mu\text{g/mL}$) for 10 min at 37°C . Samples were then centrifuged and the supernatant was stored at 4°C until further use. The bacterial pellet was suspended in lysis buffer (EDTA 4 mM, Tris 100 mM pH 7.75), and further incubated at 100°C . Cell lysates were then centrifuged and supernatants were kept on ice. The positive control is represented by the polymixin B (10 $\mu\text{g/mL}$) known to form pores in the membrane and to determine the release of ATP, while the negative control is represented by the ampicillin (50 $\mu\text{g/mL}$). Subsequently, both intra- and extra-cellular ATP levels were determined using the Molecular Probes' ATP Determination Kit, according to the manufacturer instructions (Molecular Probes' ATP Determination Kit) [24].

Membrane damage assay: DAPI/PI dual staining and fluorescence microscopy

For dual staining, 200 μL of bacterial culture (bacteria were grown to mid-logarithmic phase) was incubated in the dark for 4 h at 37 °C in agitation in the presence or absence of TiO_2 , $\text{TiO}_2\text{DOPA_ti}$ and $\text{TiO}_2\text{DOPA_polym}$ at a concentration of 200 $\mu\text{g/mL}$. After the incubation, 10 μL of bacterial culture was mixed with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) solution (1 $\mu\text{g/mL}$ DAPI final concentration) and propidium iodide (PI) 20 $\mu\text{g/mL}$. Samples were observed using an Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan) using a DAPI filter (excitation/emission: 358/461 nm). Standard acquisition times were 1000 ms for DAPI/PI dual staining. Images were captured using an Olympus DP70 digital camera [25].

DPPH radical scavenging assay

The antioxidant properties of DOPA-based/ TiO_2 nanostructures were investigated by means of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay [26]. Briefly, 100 μL of a suspension of DOPA-based/ TiO_2 nanostructures at different concentrations (from 250 to 1000 $\mu\text{g/mL}$) were added to 900 μL of a freshly prepared methanolic solution (0.002 % w/v) of DPPH. The mixtures were kept in the dark, at room temperature for 30 min. The reduction of DPPH concentration was quantified by measuring the absorbance at 515 nm. Control experiments were carried out by using TiO_2 nanostructures in the same conditions and concentration's ranges; bare DOPA was taken as positive control. DPPH scavenging activity was expressed as IC₅₀, i.e. the minimal sample's concentration able to afford 50 % decrease of initial concentration of the free radical [27]. All the experiments were run in duplicate.

Cytotoxicity on eukaryotic cell cultures

HaCat (Human keratinocytes) cells are a spontaneously transformed aneuploid immortal keratinocyte cell line from adult human skin, widely used in scientific research. These cells were maintained in Dulbecco Modified Eagle Medium (DMEM), supplemented with 10 % fetal bovine serum and 1 % penicillin-streptomycin. Cells were cultured at 37 °C in humidified atmosphere of 5 % CO_2 . The compounds were used for treatment at increasing concentrations in complete growth medium for the cytotoxicity assay [28].

Cytotoxicity on HaCat cells was assessed by performing the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) reduction inhibition assay, the colorimetric assay for assessing cell metabolic activity. Cells were grown as previously described and plated on 96-well plates at a density of 5×10^3 cells per well, in 200 μL of medium containing NSs suspensions (50, 100, 200, 400 $\mu\text{g/mL}$) for 24 h. Then, the medium was discarded and 10 μL of a stock MTT solution was added to the cells to a final concentration of 0.5 mg/mL. After 4 h incubation the MTT solution was removed and the formazan salts were dissolved in 100 μL of 0.1 N HCl in anhydrous isopropanol. Cell survival was expressed as the absorbance of blue formazan measured at 570 nm with an automatic plate reader (Multi scan spectrum, Thermo Scientific, Waltham, MA, USA). Cytotoxicity test was performed at least 3 times. Standard deviations were always <5 % for each experiment [29].

Physico-chemical analysis

The morphology of prepared hybrid nanostructures was investigated by carrying out Transmission Electron Microscopy (TEM) analysis. Particularly, samples for TEM analysis were prepared by placing a drop of the hybrid suspensions on one side of the copper grids, 200 mesh with carbon membrane. TEM images have been taken with a TECNAI 20 G²: FEI COMPANY (CRYO-TEM-TOMOGRAPHY) microscope equipped with an Eagle 2HS camera: HT 120 KV; camera exposure time: 1 s; size 2048 $\times$ 2048.

The specific surface area (S_{BET}) and the pore volume (V_p) of nanohybrids were evaluated by generating seven-point isotherms at 77 K for N_2 adsorption (Autosorb-1, Quantachrome) using an amount of char sample capable to provide a specific surface area equal to 5 m² in the sample cell. The mesopore volume (V_{BJH}), the average pore radius (r_p) and the pore size distributions were estimated by the Barreto-Joyner-Halenda (BJH) method applied to the desorption branch of the isotherm.

Crystalline phases of the TiO_2 -based nanosystems were identified by X-ray diffraction (XRD) by using a PANalytical diffractometer with a nickel filter and $Cu K_\alpha$ radiation.

The weight loss of the dried gels as well as the nature and temperatures of the various reactions occurring while heating the samples were evaluated by a TA Instrument simultaneous thermoanalyser (SDT Q600). The TG/DTA tests were performed on 20 mg dried specimens in air (temperature range: from room temperature to 800 °C; heating rate: 10 °C × min⁻¹).

Electron Paramagnetic Resonance (EPR) experiments were carried out by means of X-band (9 GHz) Bruker Elexys E-500 spectrometer (Bruker, Rheinstetten, Germany), equipped with a super-high sensitivity probe head. The analyzed samples were put into flame-sealed glass capillaries which were coaxially inserted in a standard 4 mm quartz sample tube. Measurements were performed at ~25 °C. The instrumental settings used for the analysis were defined as follows: sweep width of 100 G; modulation frequency of 100 kHz; modulation amplitude of 1.0 G and resolution of 1024 points. The amplitude of the field modulation was preventively checked to be low enough to avoid detectable signal over-modulation. Basing on these defined acquisition parameters, EPR spectra permitted to specifically monitor the organic component. In details, two sets of EPR measurements were recorded: the first one was performed on the aqueous reacting mixtures to monitor the nanohybrids synthesis, while the second one was realized on the solid powders of the final hybrid DOPA-based/ TiO_2 nanostructures in order to investigate the chemical and structural properties of the organic moiety within the nanohybrids. Specifically, EPR spectra of liquid samples were registered with an attenuation of 15 dB and several scans, typically 64, were accumulated to improve the signal-to-noise ratio. Instead, EPR spectra of solid samples were registered with an attenuation of 25 dB to avoid microwave saturation of resonance absorption curve and few scans, typically 16, were accumulated to improve the signal-to-noise ratio. Power saturation curves were also recorded by varying the microwave power from 0.004 mW to 128 mW. For each sample, 12 different spectra corresponding to distinct values of incident powers were collected.

The quantitative analysis (i.e. *g*-factor and spin-density values) of EPR spectra was realized by means of an internal standard composed by MgO/MnO powder, inserted in the quartz tube co-axially with the analyzed samples [30]. The quantitative analysis of the EPR spectra was specifically realized by determining the signal line width, ΔB , measured as peak-to-peak distance of the first-derivative signal (instrumental output), while the determination of the Gaussian and Lorentzian contributions to the line-shape was obtained by estimating the $\Delta B_{1/2}/\Delta B$ ratio, where $\Delta B_{1/2}$ is the half-height width of the EPR absorption signal. In all the cases considered in the present work, the line shape features were estimated and reported as percentages of the Lorentzian character.

Diffuse Reflectance UV-Vis (DRUV) measurements on powders were performed by using a Jasco spectrophotometer and $BaSO_4$ as a reference in the 190–850 nm wavelengths range. On the other hand, ultra-violet-visible (UV-Vis) absorption spectra on the reactive mixtures were recorded with a Cary 100 UV-Vis spectrometer from 250 to 800 nm, placing the sample into 1 cm path-length quartz optical cuvettes. The estimated resolution was 1 nm, and the background was corrected with Milli-Q water.

Results and discussion

Antimicrobial tests

Freshly prepared TiO_2 DOPA (ti and polym), and TiO_2 (as NSs control) samples have been used for dose-response curves, incubating *E. coli* (Fig. S1) and *S. aureus* (Fig. S2) cells with different nanostructures concentrations (from 0 to 400 µg/mL) to test their activity. Differently from DHICA_melanin_ TiO_2 nanostructures

that were obtained through ceramic templated approach starting from DHICA and that displayed a strong antimicrobial activity [4, 5, 20], even after 10 min of bacteria exposure, $\text{TiO}_2\text{DOPA_ti}$ does not show any antimicrobial activity, neither on the Gram negative, nor on the Gram positive strains. Indeed, $\text{TiO}_2\text{DOPA_polym}$ nanostructures showed a slight activity only against *E. coli* but after prolonged exposure (4 h – Fig. S1 panel B). This activity slowly decreased over several days of storage and it was completely lost after 3 weeks (Fig. S3). These results opened the way to the hypothesis that, unlike the similar DHICA-melanin-based nanostructures, $\text{TiO}_2\text{DOPA-NSs}$ could not be able to target the microbial membranes and show lytic activity.

In general, some antimicrobial agents kill bacterial cells by forming pores into membranes, thus determining the leakage of small molecules, such as ATP, which is synthesized on the inner side of the membrane. To confirm that DOPA-NSs were not able to damage bacterial membrane and thus induce ATP release, we performed a double assay to detect pore formation and ATP leakage on *E. coli* bacterial strain. To this purpose, *E. coli* bacterial strain was treated with TiO_2 , $\text{TiO}_2\text{DOPA_ti}$ and $\text{TiO}_2\text{DOPA_polym}$ at fixed concentration of 200 $\mu\text{g/mL}$. We first evaluated ATP leakage using the Molecular Probes' ATP Determination Kit and polymyxin B, a powerful piercing membrane antibiotic, as positive control. After, to observe the effects of NSs on the integrity of bacterial membranes, *E. coli* cells were used and stained with DAPI, fluorescent stain for DNA, and propidium iodide, an indicator of cell membrane disruption. Experimental results showed no presence of ATP outside the microbial cells treated with different types of NSs (Fig. S4) and proved that all bacterial membranes are intact (Fig. S5), also for the sample treated with such an amount of NSs which caused mortality of more than 60 % of bacterial cells. These evidences confirmed that TiO_2DOPA nanosystems do not play a direct action against bacterial membranes.

DPPH radical scavenging assay

The antioxidant properties of the TiO_2DOPA nanostructures were investigated by assaying the scavenging of DPPH, i.e. a stable free radical currently used to assess the ability of putative antioxidant compounds to act as hydrogen donors. The ability to carry out the one-electron reduction of DPPH of the TiO_2DOPA nanostructures was measured in comparison with TiO_2 nanostructures and bare DOPA. The results are reported in Table 1 and are expressed as IC_{50} , i.e. the sample's concentration able to cause a 50 % decrease of the initial absorbance at 515 nm of the DPPH radical's solution. As shown in Table 1, the IC_{50} value for the $\text{TiO}_2\text{DOPA_ti}$ nanostructures was about 23 % of that determined for $\text{TiO}_2\text{DOPA_polym}$ ones, thus revealing a higher antioxidant ability of hybrid nanostructures not submitted to the oxidation treatment by exposure to oxygen and ammonia vapors. The potential contribution of the TiO_2 component of the nanostructures to the antioxidant of power the hybrid structures was negligible, as confirmed by a IC_{50} value that is about 220-fold and 50-fold higher than the values of $\text{TiO}_2\text{DOPA_ti}$ and $\text{TiO}_2\text{DOPA_polym}$, respectively. The scavenging activity of the bare DOPA versus the DPPH was as considered as a positive control, indeed its IC_{50} value was fivefold lower than the value measured for the hybrid DOPA-based/ TiO_2 nanostructures.

Cytotoxicity assay

Assuming a possible topical application, the NSs have been tested for their toxicity on a cell line of human keratinocytes. Cytotoxicity of NSs against eukaryotic cells was evaluated by MTT assay according to the procedure described in the method section. The cytotoxic effect of TiO_2 , $\text{TiO}_2\text{DOPA_ti}$ and $\text{TiO}_2\text{DOPA_polym}$ on

Table 1: Radical scavenging activity of $\text{TiO}_2\text{-DOPA}$ based nanostructures in comparison with TiO_2 nanostructures and bare DOPA.

Sample	$\text{TiO}_2\text{DOPA_ti}$	$\text{TiO}_2\text{DOPA_polym}$	TiO_2	DOPA
DPPH IC_{50}	$2.34 \pm 0.07 \mu\text{g/mL}$	$9.95 \pm 0.15 \mu\text{g/mL}$	$506 \pm 15 \mu\text{g/mL}$	$0.468 \pm 0.003 \mu\text{g/mL}$

Values for hybrid systems are normalized to the content of organic phase (5 % w/w).

human cell lines HaCat (keratinocytes) has been verified. Cells were treated with NSs at various concentrations (from 0 to 400 $\mu\text{g/mL}$) for 24 h, and no toxicity was appreciated (Fig. S6).

Physico-chemical features of DOPA-based/ TiO_2 nanostructures

The morphology of DOPA-based/ TiO_2 nanostructures was defined by TEM images, as shown in Fig. 2. Bare TiO_2 (Fig. 2a) showed rod-like structures (3×12 nm in size), while TiO_2 /DOPA nanostructures (Fig. 2b–c) presented a rounded shape of ~ 10 nm in size due to a DOPA involvement in the growth of hybrid nanoparticles. No differences were detected for the two TiO_2 /DOPA_ti and TiO_2 -DOPA_polym systems, as far as shape and size are concerned, suggesting the morphology of nanomaterials to be defined by the hydrothermal synthesis and not influenced by the post-oxidative step.

Both TiO_2 /DOPA_ti and TiO_2 /DOPA_polym nanostructures showed a surface area of $150 \text{ m}^2/\text{g}$ as estimated by BET analysis, appearing slightly lower than that observed for bare TiO_2 ($175\text{--}190 \text{ m}^2/\text{g}$) [4, 5, 20]. These evidences confirmed that the oxidative treatment by exposure to an oxidizing atmosphere (e.g. oxygen atmosphere and ammonia vapors) did not cause changes in the porous character of the nanostructures, differently to what observed in the case of TiO_2 /DHICA_polym nanosystems [4, 5, 20]. By a structural point of view, XRD patterns of the hybrid samples, reported in the Fig. S7, are in agreement with the standard anatase profile (JCPDS 84-1286) both in terms of peak positions (i.e. the diffraction angles 2θ) and relative peak intensities [31], confirming the presence of TiO_2 crystallized in the anatase structure during the hydrothermal treatment, similarly to that observed for TiO_2 /DHICA nanosystems [4, 5, 20]. At the same time, TG analysis also allowed to determine the organic content in the nanohybrids, which was equal to 5 % w/w for both TiO_2 /DOPA_ti and TiO_2 /DOPA_polym nanosystems, also confirming no chemical change to be induced by the additional oxidation process.

The nature of the organic moiety was also investigated by EPR measurements, also providing information on its supramolecular organization within the hybrid nanostructures. Experimental EPR spectra of DOPA-melanin and TiO_2 /DOPA are reported in Fig. 3, while the spectral parameters calculated from their analysis are summarized in Table 2. DOPA-melanin spectrum shows a single and roughly symmetric EPR signal with a linewidth $\Delta B = 4.8 \pm 0.2 \text{ G}$ at a g value of 2.0041 ± 0.0003 , indicating a preferential localization of unpaired electrons on oxygen atoms of *o*-semiquinone moiety as reported in literature for synthetic DOPA-melanin [32, 33]. A high value of free radical concentration of $\sim 2.0 \times 10^{19} \text{ spin/g}$ was obtained.

On the other hand, EPR spectrum of TiO_2 /DOPA sample showed a broader single peak at a g -factor of 2.0035 ± 0.0003 (Fig. 3A), typical of carbon-centered free radicals, as observed for DHICA and DHI-derived melanins [4, 5, 9, 20, 34] and phenolic polymers [34]. The same behavior was observed for all samples obtained before (TiO_2 /DOPA_ti) and after (TiO_2 /DOPA_polym) the additional oxidative treatment (Fig. S8). The broadness of the EPR spectra of TiO_2 /DOPA nanohybrids was confirmed by the higher ΔB , equal to $6.9 \pm 0.2 \text{ G}$ with respect

Fig. 2: TEM micrographs of bare TiO_2 (a), TiO_2 /DOPA_ti (b) and TiO_2 /DOPA_polym (c) nanohybrids (scale bar 50 nm).

Fig. 3: In *panel A*, EPR spectra of DOPA-melanin (continuous line) and TiO₂DOPA_polym (dotted line). In *panel B*, plot of normalized amplitude vs. power intensities of free radicals of DOPA-melanin (full circles) and TiO₂DOPA_polym (open circles) nano hybrids.

Table 2: EPR spectral parameters for DOPA-melanin and TiO₂DOPA nanostructures.

	$\Delta B/G (\pm 0.2)$	Lorentzian %	$\text{Spin} \times g^{-1} \times 10^{19} (\leq 10 \%)$
DOPA-melanin	4.8	83 %	2
TiO ₂ DOPA	6.9	80 %	0.0018

to the DOPA-eumelanin one, as reported in Table 2 for TiO₂DOPA nanosystems, and in Table S1 for the other samples prepared with different DOPA amounts. A deeper analysis of the EPR spectra was obtained by the determination of the Lorentzian and Gaussian contributions to the line shapes, as described in the methods section. For both systems, a prevalent Lorentzian character was observed (Table 2), suggesting a substantial chemical homogeneity in the sites stabilizing free radical centers.

Finally, EPR spectra of the same samples were acquired setting an increasingly higher microwave power in order to build the power saturation profiles related to the relaxation times of paramagnetic centers present in the sample. The results are reported in Fig. 3B. The amplitude of DOPA-melanin EPR spectra increases with increasing the microwave power, reaching a maximum and then decreasing for higher microwave powers. This evidence is consistent with a homogeneous distribution of the paramagnetic centers in the sample. In contrast, the power saturation curves of TiO₂DOPA samples (Fig. 3B and Fig. S9) show an increasing trend. Thus, the presence of TiO₂ influences the DOPA behavior and organization, causing an apparent loss of the typical homogeneous distribution of free-radicals in DOPA-melanin associated with a broadening of the signal amplitude in the TiO₂DOPA spectra. A similar behavior was observed for all samples prepared with different TTiP/DOPA ratios and for samples before and after the final oxidative step (Fig. S9). This evidence, together with the different ΔB trend, suggests a strong heterogeneity in the spatial distribution of paramagnetic centers due to the TiO₂ presence, which affects the DOPA polymerization process and hinders the pigment formation. A further confirmation of this comes from the lower concentration of radical centers (magnitude order of 10³) detected in TiO₂DOPA nanosystems with respect to DOPA-melanin.

Figure 4 shows DRUV spectra of DOPA-melanin (a), TiO₂DOPA_polym (b) and bare TiO₂ (c).

Specifically, Fig. 4c shows an intense absorption in the UV range (λ < 400 nm) typical of TiO₂ phase. Furthermore, L-DOPA melanin features (Fig. 4a) a broad absorption in the whole UV-VIS spectrum as a fingerprint of melanin-like pigments. Indeed, TiO₂DOPA samples even display a continuous absorption under UV-Vis irradiation, which is more pronounced between 200 and 400 nm, due to TiO₂ presence and in the range 400–500 nm probably associated to the formation of DOPA-based polyphenols. However, the spectrum

Fig. 4: Diffuse reflectance UV spectra of DOPA-melanin (a), $\text{TiO}_2/\text{DOPA_polym}$ (b) and bare TiO_2 (c).

shows a decreasing trend in the UV region, differing from the increasing absorption of bare DOPA-melanin, thus suggesting the absence of a polyindolic backbone, typical of melanins.

To shed light on the molecular mechanism concerning the DOPA evolution in the presence of TiO_2 -sol, and driving the formation of DOPA-based/ TiO_2 nanostructures, a spectroscopic analysis involving UV-Vis and EPR techniques was also performed on the reacting mixtures used during the first stage of the nanohybrids synthesis. The corresponding experimental results are shown in Fig. 5.

UV-Vis spectrum of TiO_2 -sol at pH=1.5 (Fig. 5A, spectrum **a**) showed an adsorption edge at about $\lambda < 400$ nm, in the UV region, as expected for a wide band gap semiconductor [35]. Indeed, as soon as DOPA was added to acetic acid/ TiO_2 -sol, the system turned into orange/red, thus proving the immediate oxidation of L-DOPA to dopaquinone ($\lambda > 420$ nm) [36, 37]. Furthermore, no new absorption bands peaking at ca. 475 nm appeared in the spectrum at pH=7 (Fig. 5A, spectrum **c**), originating from the characteristic electronic transition of the colorful oxidized states of L-DOPA, after intramolecular cyclization. As further evidence, UV-Vis spectra of Fig. 5A are completely different from those collected on DOPA solution during its polymerization to melanin in alkaline environment (Fig. S10). These spectra clearly show an increasing broad absorption in the whole visible region as a feature of melanin optical behavior.

Fig. 5: In panel A, UV spectra of acetic acid/ TiO_2 -sol reaction mixture at pH=1.5 before (a) and after (b) DOPA addition and of acetic acid/ TiO_2 -sol/DOPA reaction mixture at pH=7.0 (c). In panel B, EPR spectra of acetic acid/ TiO_2 -sol/DOPA reaction mixture at pH=7.0 (a) and DOPA-melanin (b).

This evidence suggested that neither DOPA cyclization nor its polymerization do occur and was also confirmed by EPR results (Fig. 5B). Indeed, when DOPA monomer was added to the acetic acid/TiO₂-sol, no peaks appeared in the spectrum, differently to the roughly symmetric signal observed for DOPA-melanin [33]. These experimental evidences clearly indicate a different behavior of DOPA precursor in interacting with TiO₂-sol with respect to that observed in the case of DHICA monomer [21]. Particularly, DOPA bears catechol, amino and carboxylic groups, that are able to chelate Ti(IV) ions. Actually, previous studies proved that the amino-acid type binding with metal ions certainly occurs in acid conditions, together with catecholate-Ti(IV) complex [38, 39]. Formation of both complexes, protects functional groups from further reactions, thus preventing both DOPA intra-cyclization through Michael addition and its subsequent polymerization. This was only appreciated after hydrothermal treatment that however leads to polyphenolic oligomers rather than polyindole species, displaying the significant antioxidant activity and poorer antimicrobial efficacy. Actually, this antioxidant activity was stable over time and preserved even in harsh oxidative environment.

Summary and conclusions

The overall spectroscopic data are coherently supporting a model involving Ti-aminoacid complex as a key step in dictating the fate of TiO₂-sol/DOPA. This is particularly witnessed by the UV-Vis profiles and EPR features, which not only differs from those of melanin in terms of g-factor, but more importantly with respect to the spin-density. Notably, the spin density does not change after post-polymerization treatment which is associated to an increase of the oxidation of the material. These findings, coupled with the observed decrease in the antioxidant properties of the materials strongly support a picture where the DOPA-based polyphenols are not only generated in TiO₂-sol/DOPA mixture, but also preserved, in this system, towards a chemical evolution of the material under harsh oxidative environment. This different chemistry with respect to DHICA strongly impacts on the antimicrobial activity which is actually undetectable in TiO₂DOPA nanosystems, differently from what observed for hybrid TiO₂DHICA ones and prepared by the same bioinspired synthetic approach.

This study highlights the strategic role of metal complexes with melanin precursors in dictating the paths of melanogenic pathway, ultimately defining their biological functions. Furthermore, it unveils the high potential of ceramic templated approach in combination with the selection of melanin precursor in achieving a fine tuning of physical-chemical as well as bioactivity of melanin-TiO₂ nanostructures, opening to further developments towards specifically engineered biomaterials.

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