Preliminary version – not for citation

Biomacromolecules 20, 2019

<https://doi.org/10.1021/acs.biomac.9b00198>

Enzymes-lignin nanocapsules are sustainable catalysts and vehicles for the preparation of unique polyvalent bio-inks

Eliana Capecchi,¹ Davide Piccinino,¹ Bruno Mattia Bizzarri,¹ Daniele Avitabile,² Claudia Pelosi,³ Claudia Colantonio,³ Giuseppe Calabrò,³ Raffaele Saladino^{1,}*

¹ Department of Biological and Ecological Sciences (DEB), University of Tuscia, Via S. Camillo de Lellis snc, 01100 Viterbo, Italy.

² IDI Farmaceutici srl, Via dei Castelli Romani 83/85, 00040 Pomezia (Rome), Italy.

³ Department of Economics, Engineering, Society and Business Organization (DEIM), Tuscia University, Viterbo, Italy.

*Correspondence: saladino@unitus.it

KEYWORDS. Bio-Ink, lignin nanocapsules, melanin, nanostructured device, enzymatic pigmentation

ABSTRACT Reactive lignin nanocapsules catalyze pigmentation reaction to furnish an innovative type of sustainable polyvalent bio-ink. In this nano-device the pigment, vehicle, binder and additive are included in a single confined spherical space. Bio-inks with different shades of color,

black, grey, yellow-like, pink-like and red/brown hues, have been prepared by selecting the reactants and the pigmentation process. Lignin nanocapsules play multiple functions in the support and activation of the enzyme necessary for the synthesis of pigments. Lignin nanocapsules protected the melanin pigment from alkaline and UV-degradation treatment.

INTRODUCTION

Over the last years, increased legislative attention and safety burden, as well as environmental concerns, oriented traditional printing ink formulations towards the use of renewable resources with reduced or absent environmental impact ¹. Bio-inks might not only limit dependency on fuel-oil, but they also can drive the development of novel technological applications ². Ink formulations are usually composed by four type of ingredients, namely pigment, vehicle and binder, additive and solvent ³. Moreover, depending on the specific application and on the viscosity of the binder, no additional solvent might be necessary, leading to solvent free ink ⁴. Water can be used as sustainable solvent in environmental friendly ink formulations ⁵. Tall oil rosin (colophony) ⁶, soybean and edible vegetable oils ⁷, frying oils ⁸, karanja oil ⁹, abietic acid polymers ¹⁰, and polysaccharides ¹¹ have all been used as binders to replace fuel-oil derived synthetic materials. Unfortunately, drawbacks are envisaged in the application of these bio-binders, since they are in competition with food production, demanding toxic additives to improve rheological properties ¹².

Lignin, the main by-product of pulp and paper industry and of bio-refinery processes, is a sustainable binder due to its favorable physical-chemical properties associated to low cost and easy availability ¹³. Lignin is a highly branched polyphenolic polyether consisting of oligomeric sub-units produced by radical polymerization of monolignols, *para*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol ¹⁴. This polymer shows antioxidant activity ¹⁵⁻¹⁸, UV-shielding

protection ¹⁹⁻²¹, anticorrosion effects ²²⁻²³, and electrochemical responsiveness ²⁴⁻²⁶. Amine salts of Kraft lignin are additives in water-based pigments, acting both as grinding agents for the pigment in the formulation, and as binder in the printing processes ²⁷. Moreover, organosolv lignin has been applied as filler in different ink formulations, improving the viscosity of the medium ²⁸, as well as in sustainable formaldehyde resins ²⁹. In a similar way, nitrate alkaline lignin esters ³⁰, carboxylate softwood lignin ³¹, and acetylated lignin³² have been used as binders in ink formulations. Nanoparticles and nanocapsules of lignin showed innovative pharmaceutical and technological applications ³³⁻³⁵. Nano-scaled binders are of relevant interest, since they can penetrate easily into the structure of bulky paper promoting the rate of biodegradation ³⁶. For this reason, sub-micron Kraft lignin particles have been applied as binders in novel water ink formulations ³⁷. The main advantage of lignin nanodevices resides in the high biocompatibility, in the possibility to be degraded by microorganisms and in the emergence of novel scale-dimensional chemical and physical properties ³⁸.

Improved UV-shielding properties and antioxidant activities emerged during the preparation of organosolv lignin and lignosulphonate nanocapsules by a template Layer by Layer approach ³⁹, consisting on the alternative deposition of polyelectrolytes of different electrical charge⁴⁰. Lignin nanodevices have been used as support for the immobilization of enzymes⁴¹, including tyrosinase and laccase⁴². In this latter case, they showed capacity to improve the enzymatic activity by boosting effect ⁴³ and by direct and mediated electron transfer processes ⁴⁴. Tyrosinase and laccase catalyze the preparation of several pigments and dyes under natural or synthetic procedures ⁴⁵. Thus, lignin nanocapsules functionalized with oxidative enzymes can perform as biocatalyst for the synthesis of the pigment, and as vehicle, binder, and additive for the newly synthesized pigment. Here we describe the use of lignin nanocapsules loaded with

tyrosinase from *Aspergillus niger* and, in alternative, with laccase from *Trametes versicolor*, for the efficient “in situ” polymerization of appropriate chemical precursors to afford a large panel of pigments. The pigments resulted to be stably linked to the support affording a large panel of colored nanocapsules to be used as a unique polyvalent bio-ink.

EXPERIMENTAL PART

Materials

Organosolv lignin (OL) was purchased from Chemical Point (Oberhaching, Germany). Tyrosinase from *Aspergillus niger* (1000 U/mg), laccase from *Trametes versicolor* (1.07 U/mg), Bradford reagent, poly(diallyldimethylammonium chloride) (PDDA, 20%v/v water solution), chitosan (CH, low molecular weight) with 20% chitin units, melanin, 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), L-tyrosine, L-DOPA, epicatechin, catechol, 4-amino benzoic acid, hydroquinone, 3-aminobenzoic acid, resorcinol, and *para*-phenylenediamine were purchased from Sigma–Aldrich (St. Louis, MO, USA), and were used without any further purification. All experiments were repeated in triplicate using native and immobilized tyrosinase or laccase in phosphate buffer saline (PBS 0.1 M pH 7) or sodium acetate buffer (Na-acetate buffer 0.1 M pH 5), respectively. Solutions were prepared using Milli-Q water ($\rho = 18.2 \text{ M}\Omega \text{ cm}$ a 25 °C; $\text{TOC} < 10 \text{ }\mu\text{g L}^{-1}$, Millipore, Molsheim, France).

Preparation of nanocapsules by a template-based Layer-by-Layer procedure

Nanocapsules were prepared by applying a modified version of the Layer-by-Layer (LbL) procedure using organosolv lignin (OL) as template⁴⁶. Briefly, commercially available OL (20 mg) was dissolved in THF (20 mL), followed by filtration (0.45 μm syringe filter) and dialysis against deionized water, for 24h at 25°C. The suspension (15 mL) of OL nanocapsules in MilliQ water (1

mg/ml; pH 4.2) was added dropwise to PDDA or CH solution (1.0 mg/mL, MilliQ water) under slow magnetic stirring ⁴⁷. After 2 h, the final suspension was centrifuged (6000 rpm, 20 min) and the supernatant removed. The nanocapsules were rinsed with deionized water, recovered by centrifugation (6000 rpm, 20 min) and lyophilized to yield OL/PDDA and OL/CH intermediates, respectively.

Procedure for the loading of tyrosinase

The reactivity of tyrosinase towards lignin is reported⁴². As a general trend, tyrosinase showed negligible activity towards lignin modification⁴⁸. Tyrosinase from *Aspergillus niger* (1.0 mg, 900 U/mg) was added to a suspension of OL-PDDA (100 mg), or in alternative, to OL/CH (100 mg), in the appropriate buffer (100 mL) under orbital shaking for 24h at 25°C. OL/PDDA/tyrosinase (**I**) and OL/CH/tyrosinase (**II**) were recovered by centrifugation (20 min at 6000 rpm). Compounds (**I**) and (**II**) were washed several times with PBS in order to ensure the complete removal of unbounded enzyme (as evaluated by the Bradford method). The recovered solution was used for the evaluation of the activity parameters.

Procedure for the loading of laccase

Laccase from *Trametes versicolor* (20 mg, 1.07 U/mg) was added to a suspension of OL-PDDA (100 mg), or in alternative, to OL/CH, in Na-acetate buffer (100 mL) under orbital shaking for 24h at 25°C. OL/PDDA/laccase (**III**), and OL/CH/laccase (**IV**), were recovered by centrifugation (20 min at 6000 rpm). Compounds (**III**) and (**IV**) were washed several times with Na-acetate buffer in

order to ensure the complete removal of unbounded enzyme (as evaluated by the Bradford method). The recovered solution was used for the evaluation of the activity parameters.

Determination of the kinetic parameters

Kinetic parameters of compounds (**I-II**) (K_m , V_{max} , and V_{max}/K_m) were determined spectrophotometrically by measuring the activity of immobilized tyrosinase at different concentrations of L-tyrosine (0.33–0.10 mM) using the dopachrome method⁴⁹. As a general procedure, the reaction was started by adding L-tyrosine to PBS solution (0.1 M, pH 7) containing the appropriate amount of compounds (**I**) and (**II**) (0.1 mg). The initial rates were measured in a 3.0 mL reaction cuvette as linear increase of the optical density (475 nm) at 25°C under magnetic stirring.

Kinetic parameters of compounds (**III**) and (**IV**) were determined by measuring the activity of immobilized laccase at different concentrations of ABTS (0.1–5 mM). Briefly, the reaction was started by adding ABTS to compounds (**III**) and (**IV**) (0.1 mg) in Na-acetate solution (0.1 M pH 5). The initial rates were measured in a 3.0 ml reaction cuvette as the linear increase of the optical density at 436 nm at 25°C under magnetic stirring. The data were analyzed by a double reciprocal Lineweaver–Burk plot. Spectrophotometric data were obtained by using a Cary WinUV (AGILENT, Santa Clara, CA, USA). All experiments were conducted in triplicate using free and immobilized tyrosinase and laccase.

Determination of activity parameters.

The activity parameters of tyrosinase were determined by measuring the tyrosinase catalyzed oxidation of L-tyrosine by the dopachrome method. As a general procedure, the reaction was started by adding L-tyrosine (0.83 mM) to PBS solution (67 mM, pH 7) containing the appropriate amount (from 45 to 100 units) of samples (**I-II**) (from 0.045 to 0.1 mg). The initial rates were measured in a 3.0 mL reaction cuvette as the linear increase of the optical density (475 nm) at 25 °C under magnetic stirring. One unit of enzyme activity was defined as the increase in absorbance of 0.001 unit per minute at pH 7 and 25 °C. Spectrophotometric data were analyzed with Cary WinUV. All experiments were conducted in triplicate. The activity parameters of samples (**III-IV**) were assayed by using the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) procedure. ABTS (5 mM), sodium acetate buffer (2.0 mL, pH 5.0), and the sample solution (200 µL) were used as standard solution. The formation of the cation radical was detected by measuring the increase of absorbance at 436 nm. One unit of laccase activity has been defined as the amount of enzyme that catalyzed the oxidation of 1.0 µmol of ABTS in 200 µL reaction mixture at 25 °C during 1.0 min.

Dynamic light scattering

Measurements of particle size and Zeta potential were performed on freshly prepared aqueous suspensions (pH = 7) of OL nanocapsules and compounds (**I-II**) were dispersed in water by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) apparatus, equipped with an He-Ne laser (633 nm, fixed scattering angle of 173°, 25°C). Prior to perform size measurements, the suspensions were filtered with a 0.45 µm filter membrane. Measurements were performed in triplicate at room temperature (T = 25 °C).

Procedure for the pigmentation of lignin nanocapsules functionalized with tyrosinase

As a general procedure, OL/CH/tyrosinase (**II**) (1.0 mg/mL) was suspended in PBS buffer (pH 7, 0.1 M) in the presence of L-DOPA PBS solution (2 mM) and the suspension was stirred for 2 hours at 25°C under oxygen atmosphere. The black suspension was centrifuged (6000 rpm, 20 min) and the supernatant removed. Nanocapsules were rinsed three times with KOH (1.0 M) in order to ensure the complete removal of unbounded melanin, followed by washing with deionized water up to neutral pH. Next, nanocapsules were recovered by centrifugation (6000 rpm, 20 min) and lyophilized to yield compound (**V**). The amount of unbounded melanin in the KOH solution was evaluated spectrophotometrically by comparing the OD at 470 nm of unknown sample with a standard melanin (1 mg/ml)⁵⁰. All measurements were done in triplicate. The disappearance of L-DOPA during the reaction was monitored by gas-chromatography associated to mass-spectrometry (GC-MS). The analyses were performed by 450 GC-320 MS apparatus using a VF-5MS column and an isothermal temperature profile of 100 °C for 2 min, followed by a 10 °C/min temperature gradient to 280 °C for 25 min. The injector temperature was 280 °C. Chromatography-grade helium was used as the carrier gas with a flow of 2.7 mL/min. Mass spectra were recorded with an electron beam of 70 eV.

Procedure for the pigmentation of lignin nanocapsules functionalized with laccase

As a general procedure, OL/CH/Laccase (**IV**) (1.0 mg/ml) was suspended in Na-acetate buffer (pH 5, 0.1M) in the presence of the appropriate substrate (2.0 mM) in 0.1 M sodium-acetate buffer (pH 5.0), containing 2% (v/v) absolute ethanol ⁴⁵. The suspension was stirred for 24 hours at 25°C under oxygen atmosphere. The final suspension was centrifuged (6000 rpm, 20 min) and the supernatant removed. The product was rinsed three times with MilliQ water (10 mL) in order to ensure the complete removal of unbounded pigment. Residual traces of unreacted substrate were monitored by GC-MS. Irrespective to experimental conditions, the quantitative conversion of the substrate was observed.

The colored nanocapsules were recovered by centrifugation (6000 rpm, 20 min) and lyophilized to yield compounds (**VIII**), (**IXa-IXb**), (**Xa-Xb**), and (**XIa-XIb**). The formation of the epicatechin polymer with OL/CH/Laccase (**IV**) was evaluated spectrophotometrically in the range of 250–300nm at different reaction times (1 h, 2 h and 6 h) using epicatechin monomer as standard.

Electron microscopy analysis

For Scanning Electron Microscopy (SEM), compounds (**V-VIII**), (**IXa-IXb**), (**Xa-Xb**), and (**XIa-XIb**) were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), washed and post-fixed in OsO₄ (1% weight) in the same buffer at room temperature. Specimens were dehydrated in a graded ethanol series. They were then air dried, sputter-coated with gold in a Balzers MED 010 unit. The observation was made by a JEOL JSM 6010LA electron microscope. For Transmission Electron Microscopy (TEM), nanocapsules were placed on formvar-carbon-coated grids and allowed to be adsorbed for 60 s. Excess liquid was gently removed from each droplet with a strip of filter paper. Processing for negative staining was carried out by placing a drop of negative stain (2%uranyl acetate in distilled water) on the specimen grids, followed by quick blotting. This step

was repeated a second time on a new drop of negative staining solution and prolonged for 60 s. Nanocapsules were observed with a JEOL 1200 EX II electron microscope. Micrographs were acquired by the Olympus SIS VELETA CCD camera equipped the iTEM software.

FTIR Characterization

Attenuated total reflection infrared (ATR-IR) spectroscopic analyses were performed, at room temperature, with a Perkin–Elmer Spectrum One spectrometer equipped with an ATR-IR cell. IR spectra were recorded by averaging 32 scans, with a resolution of 4 cm⁻¹.

EPR analysis

EPR spectra of solid samples were recorded in a CW (Continuous wave) X-band (9GHz) at room temperature with a Bruker E580 Elexsys Series using the Bruker ER4122SHQE cavity. The 1mm diameter quartz capillaries were filled in with the samples and they were placed into a suprasil tube of 3.5 mm internal diameter. Experimental conditions for acquisition were: 9.87 GHz microwave frequency, 0.1mT modulation amplitude and 2mW microwave power.

Characterization of color parameters after pigmentation of lignin nanocapsules

Color parameters were measured through X-Rite CA22 reflectance spectrophotometer according to the CIE L*a*b* color system (ISO 11664-4) ⁵¹. In this system, the coordinate L* describes the lightness, while the coordinates a* and b* describe the chromatic coordinates on the green-red and

blue-yellow axes, respectively. The characteristics of the color measuring instrument are the following: light source D65; standard observer 10°; fixed geometry of measurement 45°/0°; spectral range 400-700 nm; spectral resolution 10 nm; aperture size 4 mm. For each sample three measurements were performed, and then average values and standard deviations were calculated. Data are reported as L*, a*, b*, and reflectance spectra in the visible range.

Effect of alkaline treatment and of Ultraviolet photo-irradiation on the color strength and fastness properties

The effect of UV irradiation, on the color change of compounds (V) and (VIII) in lyophilized powder (60 mg) on a glass slide (size 25 mm × 75 mm) support was performed by UVA and UVB light irradiation, which was provided by UV LAMP 300 W (ULTRAVITALUX, UVA power 13.6 W and UVB power 3.0 W). After 2 or 24 h of light irradiation the compounds (V) and (VIII) were collected for the characterization of color parameters using the untreated compounds as reference. As reported in literature, the effect of the treatment with NaOH (1.0 M) on the color change of compound (V) was performed by using a slightly modified procedure⁵². After 2 or 24 h of NaOH (1.0 M) treatment the final suspension was centrifuged (6000 rpm, 20 min) and the supernatant removed. The compound (V) was lyophilized for the characterization of color parameters using the untreated compound V as reference.

RESULTS AND DISCUSSION

Preparation of lignin nanocapsules and their loading with tyrosinase and laccase

Functionalized lignin nanocapsules supporting tyrosinase and laccase have been prepared by a modified version of the Layer-by-Layer (LbL) procedure (Scheme 1, pathway A), consisting in the consecutive deposition of alternatively charged polyelectrolytes onto the appropriate support⁵³. The coating protects the enzyme from denaturing agents and allow regulation of the permeability towards small substrates⁵⁴. Briefly, nanocapsules of organosolv lignin (OL) were obtained by dialysis of a lignin tetrahydrofuran (THF) solution against deionized water⁴⁶. The OL nanocapsules were used as a template for the sequential deposition of positively charged poly (diallyl dimethylammonium chloride (PDDA) and negatively charged tyrosinase from *Aspergillus niger*. Kinetic parameters of OL/PDDA/tyrosinase (**I**) (K_m , V_{max} and V_{max}/K_m), determined by measuring the enzyme activity at different concentrations of L-tyrosine, were found to be in agreement with data previously reported⁴² (Table 1, entry 1), confirming the effective loading of the enzyme.

Scheme 1. Structure model of lignin,⁵⁵ and schematic preparation of functionalized lignin nanocapsules supporting tyrosinase and laccase. PDDA and CH were used as positively charged polyelectrolytes. Tyrosinase from *Aspergillus niger*, and laccase from *Trametes versicolor*, are negatively charged at the operative pH. The consecutive deposition of alternatively charged polyelectrolytes was performed on the surface of organosolv lignin nanocapsules.

Table 1.

Kinetic parameters of functionalized lignin nanocapsules (I-IV).

Entry	Sample	K_m (mM)	V_{max} ($\times 10^{-3}$) ^c (Δ Abs min μ g enzyme)	$V_{max}/K_m \times 10^{-3}$	Immobilization Yield (%) ^d
1	(I) ^a	0.37	3.89	10.51	90.0
2	(II) ^a	0.36	3.88	10.77	89.5
3	(III) ^b	0.17	1245	7323	64.1
4	(IV) ^b	0.16	1240	7750	62.3

^a Tyrosinase based system. L-tyrosine was used as a substrate.^b Laccase based system. ABTS was used as a substrate.^c V_{max} represents the maximum rate achieved by the system, at a saturating substrate concentration. The Michaelis constant K_m is the substrate concentration at which the reaction rate is half of V_{max} . All experiments were conducted in triplicate. Average errors in kinetic parameters were 2–4% for K_m , and 1–3% for V_{max} .^d Immobilization yield (%) = $[(U_a - U_r)/U_a] \times 100$. U_a is the total activity (units) of the enzyme added to the solution, and U_r is the residual activity (units) in the washing solutions.

Chitosan (CH), a linear polysaccharide composed of randomly distributed β -(1,4)-D-glucosamine and *N*-acetyl-D-glucosamine units, was successfully used as biodegradable and no toxic alternative to PDDA ⁵⁶ to yield OL/CH/tyrosinase (II). As reported in Table 1 (entry 2), the kinetic parameters of OL/CH/tyrosinase (II) were of the same order of magnitude than that of OL/PDDA/tyrosinase (I).

The scanning electron microscopy (SEM) image of compound (II) showed nanoparticles with a regular spherical shape and an average diameter of $0.22 \pm 0.02 \mu\text{m}$ (Figure 1). [SEM image of compound (I) is reported in Supporting Information SI#1]. Dynamic Light Scattering (DLS) analysis of compounds (I-II) is reported in Figure 2.

Figure 1. SEM images of lignin nanocapsules. Panel A: organosolv lignin nanocapsules. Panel B: OL/CH/tyrosinase (**II**).

Figure 2. Size distribution and polydispersity index (PDI) of OL nanocapsules (blue line, PDI=0.349), compound (**I**) (red line, PDI=0.353), and compound (**II**) (orange line, PDI=0.382).

In order to improve the range of catalytic activity, laccase from *Trametes versicolor* was used instead of tyrosinase as a negative layer to yield OL/PDDA/laccase (**III**) and OL/CH/laccase (**IV**), respectively (Scheme 1, pathway B). Kinetic parameters of compounds (**III**) and (**IV**) are reported in Table 1 (entries 3 and 4). Compounds (**III**) and (**IV**) showed kinetic parameter higher than that measured for similar catalysts previously produced by LbL immobilization of laccase on Multi Walled Carbon Nanotubes (MWCNTs)⁵⁴. SEM images of compounds (**III**) and (**IV**) are in

SI# 2. The immobilization yield of samples (**I-IV**) was evaluated as reported in the following equation (Table 1):

$$\text{Immobilization yield (\%)} = [(U_a - U_r)/U_a] \times 100$$

Where U_a is the total activity (units) of the enzyme added to the solution, and U_r is the residual activity (units) in the washing solutions.

Procedure for the pigmentation of lignin nanocapsules functionalized with tyrosinase

Tyrosinase (EC 1.14.18.1) catalyzes the conversion of L-tyrosine, and L-3,4-dihydroxyphenylalanine (L-DOPA), to melanin⁵⁷, which is a black pigment widely used in bio-ink formulations⁵⁸. The mechanism for the synthesis of melanin, especially for the most common variant, eumelanin, is reviewed⁵⁹. It comprises the formation of colored reactive intermediates such as dopachrome, followed by sequential oxidative coupling reactions.

Eumelanin is usually represented as a complex structure with oligomeric chain lengths of up to a few tenth of monomers and variable chemical composition⁶⁰. Synthetic melanin is mostly reported as amorphous solid without distinctive particles⁶¹, although some evidences exist that spherical particles can be produced from synthetic sources under controlled conditions⁶². In this latter case, a three-fold aggregation of oligomers, based on π -stacking interactions, have been proposed⁶³.

With the aim to synthesize melanin on the surface of lignin nanocapsules, we decided to use OL/CH/tyrosinase (**II**) as a representative sample, being this nanodevice composed by bio-based ingredients. OL/CH/tyrosinase (**II**) (1.0 mg/mL) was suspended in PBS buffer (pH 7, 0.1

M) in the presence of L-DOPA (10 mg), and the suspension was stirred at 25°C under oxygen atmosphere. The system showed a red coloration, probably due to the formation of the dopachrome intermediate, followed by the appearance of the characteristic black coloration of melanin. L-DOPA was completely converted after 2.0 h of reaction time (as evaluated by gas-chromatography associated to mass spectroscopy analysis) to yield black lignin nanocapsules (V). Nanocapsules (V) retained the black coloration after treatment with KOH (1.0 M, 3 x 1.0 mL), suggesting a stable linkage between newly synthesized melanin and the lignin support (Scheme 2, pathway A). Lignin nanocapsules were stable during the KOH treatment, in accordance with previously reported data on the increased stability of lignin nanoparticles at high pH value, as a consequence of the structuring process⁶⁴. Moreover, the alkaline stability of lignin nanoparticles was found to be significantly improved after chemical grafting with dopamine, due to cross-linking reticulation reactions⁶⁵. Tyrosinase catalyzes the oxidative coupling between phenolic units (even when sterically hindered) by formation of reactive quinone intermediates⁶⁶, that can diffuse towards chitin layer to lignin in accordance with results previously reported on the diffusion- and reaction rate-limited redox processes mediated by quinones through bilayer lipid membranes⁶⁷.

Pathway

Scheme 2. Schematic representation of the synthesis and subsequent deposition of melanin in the reaction of OL/CH/tyrosinase (II) with L-DOPA and L-DOPA/L-tyrosine mixtures, respectively. Different shades of gray were obtained depending on the L-DOPA/L-tyrosine ratio.

This reaction mechanism might be responsible for the formation of covalent bonds between the phenolic units of lignin and the growing melanin oligomers, assuring the observed stability of the black pigment on the surface of compound (V). π -Stacking interactions between the aromatic moieties of the lignin and melanin may be also responsible for the stability of the nanodevice⁶³.

TEM and SEM investigations of compound (V) (Figure 3 and Figure 4, respectively) showed the deposition of distinguishable agglomerates of melanin on the surface of lignin. The irregular shape of these agglomerates, as highlighted by the TEM analysis, suggested the presence of a variable number of superimposed layers of amorphous melanin. Note that spherical nanoparticles of melanin have been previously obtained using tyrosinase adsorbed on an inert glass surface, in which case interactions were not operative between melanin and the support⁶⁸.

Figure 3. TEM images of lignin nanocapsules. Panel A: Organosolv lignin nanocapsules. Panel B: Black lignin nanocapsules (V). Agglomerates of amorphous melanin are clearly evident on the surface of lignin.

Figure 4. SEM images of lignin nanocapsules. Panel A: Organosolv lignin nanocapsules. Panel B: Black lignin nanocapsules (V)

Moreover, the internal spherical shape of compound (V) was similar to that of compound (II), indicating that the L-DOPA polymerization process was limited to the surface of lignin (Figure 3, panel A versus panel B).

Characterization of the melanin pigment and production of the gray color

The UV–vis-spectra of the KOH solution recovered during the preparation of compound (V) was similar to that of the standard (Figure 5), confirming the synthesis of the melanin pigment. The amount of unbounded melanin was measured by comparing the OD at 470 nm with respect to standard sample³⁵. As reported in SI# 3, 0.2 mg/mL of unbounded melanin were detected, indicating that c.a. 0.8 mg of melanin were retained per 10 mg of compound (V).

Figure 5. Comparison between the UV–vis-spectra of the KOH solution recovered during the preparation of compound (V) and the standard sample. Blue line: UV–vis-spectra of unbonded melanin produced by polymerization of L-DOPA in the presence of OL/PDDA/tyrosinase (II). Red line: UV–vis-spectra of standard melanin.

The ATR-FTIR-spectra of compound (II) showed the vibration bands for the most frequent monomeric sub-units in melanin, 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA), respectively. However, the signal of the carboxylic acid (COOH, 1038 cm^{-1}) was not found in the standard, indicating a lower DHICA content in the reference sample of melanin (Figure 6, Panel A).

Figure 6. Panel A: ATR-FTIR-spectra of melanin recovered from the KOH solution (blue line), and standard melanin (red line). Band assignment: 3196 cm^{-1} OH-, NH- and water (broad signal); 1700 cm^{-1} quinone (C=O stretching); 1606 cm^{-1} indole (ring vibration); 1440 cm^{-1} pyrrole (C=C stretching); 1250 cm^{-1} pyrrole (N-C stretching); 1038 cm^{-1} COOH pyrrole (DHICA monomer). Panel B: ATR-FTIR-spectra of OL/PDDA/tyrosinase (**II**) (red line), and compound (**V**) (blue line). The band at 1038 cm^{-1} COOH in compound (**V**), corresponding to the pyrrole group (DHICA monomer), is characteristic of the melanin structure.

The vibrational bands of melanin were also detected in the ATR-FTIR-spectrum of compound (**V**) (Figure 6, Panel B), showing the characteristic COOH absorption band, associated to the pyrrole group (DHICA monomer), at 1038 cm^{-1} .

The presence of the melanin pigment in sample (**II**) was definitely proved by first-derivative Electron Paramagnetic Resonance (EPR) analysis (Figure 7, Table 2, entry 2), which showed the characteristic melanin EPR signal at a g factor of 2.0041^{69-70} .

Figure 7. The first-derivative EPR analysis of sample (V). Red line: standard melanin. Green line: sample (V). Blue line: OL nanocapsules.

Table 2.
Mean values of g -factor of samples (V-VII).

Entry	Sample	g factor ^a
1	Standard melanin	2.0036
2	sample (V)	2.0041
3	sample (VI)	2.0042
4	sample (VII)	2.0038

^a The error on the g factors was ± 0.0001

Next, experiments using L-DOPA in the presence of different concentrations of L-tyrosine were performed in order to tune the shade of the melanin pigment⁷¹.

When L-DOPA (2.0 mM) was reacted with L-tyrosine (1.0 mM and 0.5 mM, respectively) in the presence of compound (II) under previously reported conditions, lignin nanocapsules (VI) and (VII), characterized by two different shade of gray, were obtained. The lighter shade of gray was observed in the presence of the lower amount of L-tyrosine (Scheme 1, pathways B and C), suggesting the possible control of the shade simply by varying the ratio between the reagents. SEM images of nanocapsules (VI) and (VII) are reported in SI# 4. The effective presence of melanin in compounds (VI) and (VII) was demonstrated by first-derivative EPR experiments, in which the

characteristic signal of melanin was observed at g factor of 2.0042 and 2.0038, respectively (Table 2, entries 3 and 4). Probably, the gray color was a consequence of a low concentration of the pigment.

Procedure for the pigmentation of lignin nanocapsules functionalized with laccase

Laccase (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductase) is a multicopper enzyme that catalyzes the oxidation of four molecules of substrate through the direct reduction of dioxygen (O_2)⁷². The industrial interest in the application of laccase lies in the low substrate selectivity, high value of catalytic constants, and high thermal resistance⁷³. Immobilized laccase has been also used in the chemistry of lignin⁷⁴. Laccase efficiently catalyzes the polymerization of phenol derivatives to form pigments in dyeing processes and formulations⁴⁵. In this latter case, a large panel of color and a wide range of shade have been obtained by using heteropolymer synthesis⁷⁵. For example, mixture of natural compounds belonging to the flavonoids family have been successfully applied to give rise to yellow, orange and dark brown colored heteropolymers⁷⁶.

When the flavonoid epicatechin (2.0 mM) was treated with OL/CH/laccase (**IV**) (1.0 mg/mL) in Na-acetate buffer (0.1 M, pH 5) at room temperature under dioxygen atmosphere, the yellow-like colored lignin nanocapsules (**VIII**) were obtained (Scheme 3, pathway A). The color was retained after washing with Na-acetate buffer, suggesting the occurrence of stable interactions between the epicatechin polymer and lignin. This result is in accordance with data previously reported on the laccase mediated grafting-process of flavonoids on cotton fibers⁷⁷. The mechanism for the laccase mediated formation of covalent linkages between raw lignin and flavonoids is reviewed, including the formation of C-C and C-O covalent bonds⁷⁸.

Pathway

A

B

C

D

Scheme 3. Schematic representation of the synthesis and subsequent deposition of pigments during the reaction of OL/CH/laccase (IV) with phenol derivatives.

The SEM image of compound (VIII) showed spherical capsules with an average diameter of $0.33 \pm 0.02 \mu\text{m}$ (Figure 8, Panel A), while in the TEM analysis the presence of the epicatechin layer surrounding the lignin capsule was highlighted (Figure 8, Panel B).

Figure 8. Panel A: SEM image of the yellow-like lignin nanocapsules (VIII). Panel B: TEM image of the yellow-like lignin nanocapsules (VIII).

The formation of the epicatechin polymer was monitored by UV-visible titration at different reaction times (Figure 9). Epicatechin showed a major absorption band in the range of 250–300 nm. The addition of OL/CH/laccase (IV) afforded the decrease of the initial absorption band, with the contemporary formation of a new peak in the 325–800 nm range (with a maxima at 392 nm), corresponding to expected epicatechin polymer.

Figure 9. UV-visible titration of the reaction between OL/CH/laccase (IV) and epicatechin. Newly synthesized epicatechin polymer showed a characteristic absorption peak in the 325–800 nm range.

The ATR-FTIR spectra of compound (VIII) confirmed the modification of lignin during the reaction with epicatechin, as evidenced by the disappearance and attenuation of some of the main absorption bands characteristic of lignin (1738 cm^{-1} , 1364 cm^{-1} and 1217 cm^{-1}) (Figure 10, Panel A). Moreover, the spectra showed the expected absorption bands, including the OH stretching ($3400 - 3100\text{ cm}^{-1}$), C = C stretching (1600 cm^{-1}), and C – O stretching ($1150 - 1010\text{ cm}^{-1}$)⁷⁹.

Figure 10. ATR-FTIR spectra of compounds (**VIII**) and (**IXa**). Panel A (red line): absorption bands of compound (**IV**). Panel A (blue line): absorption bands of compound (**VIII**). Panel B (red line): absorption bands of compound (**IV**). Panel B (blue line): Absorption bands of compound (**IXa**). Characteristic adsorption bands of the amino-benzoic scaffold were found at 760 cm^{-1} , 860 cm^{-1} , 1490 cm^{-1} , 1600 cm^{-1} and 1640 cm^{-1} , respectively.

A larger panel of colours was obtained by reaction of compound (**IV**) with mixtures of phenol derivatives. The treatment of catechol (1.0 mM) and 4-amino-benzoic acid (0.5 mM) with compound (**IV**) afforded pink-like colored lignin nanocapsules (**IXa-b**), whose shade intensity was found to be dependent from reaction time, the darker shade corresponding to the higher value of reaction time (Scheme 2, pathway B). The presence of catechol/4-amino-benzoic acid heteropolymer on the lignin surface of nanocapsules (**IXa**) was confirmed by the TEM analysis (Figure 11).

Figure 11. TEM image of nanocapsules (**IXa**).

ATR-FTIR spectrum of compound (**IXa**) showed typical signals for the amino-benzoic scaffold associated to the formation of the catechol/4-amino-benzoic acid heteropolymer, including frequency at 760 cm^{-1} , 860 cm^{-1} , 1490 cm^{-1} , 1600 cm^{-1} , and 1640 cm^{-1} ⁸⁰ (Figure 10, Panel B).

Red/brown-like lignin nanocapsules (**Xa-b**), and grey/black lignin nanocapsules (**XIa-b**), have been obtained by the co-polymerization of hydroquinone (1.0 mM) with 3-amino benzoic acid (0.5 mM) (Scheme 2, pathway C), and of resorcinol (1.0 mM) with *para*-phenylenediamine (0.5 mM) (Scheme 2, pathway C), respectively. Again, the darker shade of the colour was obtained in the correspondence of the highest reaction time. Pigments were found to be stably linked to lignin, confirming the formation of covalent linkages between the colored heteropolymer and the support. TEM images and ATR-FTIR analyses of compounds (**Xa**) and (**XIa**) are in SI# 5 and SI# 6, respectively.

Unexpectedly, reactions of compound (**IV**) produced also pigments that were un-bonded to lignin. For example, 2-amino-3-methoxy benzoic acid (Scheme 4, pathway A), 2-methoxy hydroquinone (Scheme 4, pathway B), and the mixture of 4-hydroxy-benzoic acid with ABTS (Scheme 4, pathway C), or in alternative, the mixture of 4-methyl-amino benzoic acid with 4-methoxy-phenol (Scheme 4, pathway D), afforded orange, yellow, blue and violet pigments that were lost during the work-up of the reaction ⁴⁵.

These data suggested that catechol or hydroquinone moieties were necessary for the formation of a stable linkage between the pigment and lignin. Probably, the higher reactivity of these derivatives with respect to simple phenols in the oxidative cross-coupling process may be responsible for the observed results ⁸¹. This hypothesis is in accordance with the role that quinone derivatives play in the covalent grafting of lignin ⁸².

Pathway

Scheme 4. Unproductive OL/CH/laccase (IV) catalyzed polymerization of phenol derivatives 2-amino-3-methoxy benzoic acid (pathway A), 2-methoxy hydroquinone (pathway B), and of mixture of 4-hydroxy-benzoic acid with ABTS (pathway C), or 4-methyl-amino benzoic acid with 4-methoxy-phenol (pathway D). After the reaction the orange, yellow, blue and violet pigments were not retained on the surface of the lignin nanocapsules.

Characterization of color parameters after pigmentation of lignin nanocapsules

The characterization of the color parameters of compounds (V-VIII), (IXa-b), (Xa-b) and (XIa-b) has been performed by reflectance spectrophotometry (Figure 12 panel A) using the CIE $L^*a^*b^*$ system (Figures 12 panel B and C). The reflectance spectrum and the values of chromatic coordinates indicated for the reference lignin nanocapsules a yellowish color (average value of b^* equal to 23.3)⁸³. Compound (V) confirmed a black-grey color having low reflectance in the visible spectrum and low values of the chromatic coordinates. Similar trend was observed in compounds (XIa) and (XIb), that exhibited, in respect to compound (V), only a slightly higher values of lightness.

Lignin nanocapsules (VI) and (VII) showed constant values in the reflectance spectrum along the entire visible range, confirming the light grey color of these samples. The two different grey shades were also documented by color measurements: compound (VI) showing the higher value of lightness and the lower value of b^* with respect to compound (VII).

Figure 12. Panel A: Reflectance spectra of colored lignin nanocapsules (V-VIII), (IXa-b), (Xa-b) and (XIa-b). Panel B: CIE $L^*a^*b^*$ parameters of colored lignin nanocapsules (V-VIII), (IXa-b), (Xa-b) and (XIa-b). Panel C: Chromatic diagram for a^*b^* parameters of colored lignin nanocapsules (V-VIII), (IXa-b), (Xa-b) and (XIa-b). Acronym S is for standard compounds (II) and (IV). Panel D: Reflectance spectra of compound (V) and (VIII) after UV irradiation and treatment with NaOH of compound (V).

Compound (VIII) exhibited a reflectance spectrum similar to that of compounds (II) and (IV), with a clear dominance of yellow, as showed by the b^* value. Compounds (IXa-b) showed a different trend in the reflectance spectra and in the values of chromatic coordinates a^* and b^* , whereas the values of L^* were the same for the two samples. In particular, compound (IXb) has a

higher value of b^* indicating a yellow dominant of the color, on the other hand, sample (**IXa**) is located very close to the center of the chromatic diagram, i.e. the grey scale axis.

Compounds (**Xa**) and (**Xb**) exhibited the typical reflectance spectra of orange-red color with a rapid increase of reflectance in the red portion of the visible range at 650-700 nm, especially highlighted in the case of compound (**Xb**).

Effect of ultraviolet photo-irradiation and alkaline treatment on the color strength and fastness properties

The effect of UV photo-irradiation on the color strength and light fastness properties was evaluated in the case of compounds (**V**) and (**VIII**) as representative examples of black colored and yellow-like colored nanoparticles. Color strength and light fastness are important physical properties for the quality, weather resistance, and applications of the inks. Alkaline medium and artificial and natural light may cause most pigments to degrade resulting in color change. The light fastness of inks is important especially in tattoo, in packaging and in printing industries⁸⁴. Light fastness refers to the resistance of a color to fading under the sunlight radiation. The effect of UV irradiation on the color change of compounds (**V**) and (**VIII**) is reported in Figure 12 (panel D) and Table 3. The color changes were defined as both variation of the single chromatic coordinates and total color variation (ΔE^*). ΔE^* is the geometrical distance between two points in the CIE $L^*a^*b^*$ colorimetric space and is calculated according to equation (1):

$$(1) \Delta E^* = \sqrt{\{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2\}}$$

Samples (**V**) and (**VIII**) showed only a slight color variation after 2 h of UV irradiation as indicated by the ΔE^* value (Table 3, entries 2 and 7). A value of ΔE^* close to 2 is considered imperceptible

by human eye, the perceptible variation being observed only at ΔE^* value close to 5⁸⁵. Interestingly, the sample (V) showed a darker black color (that is a decrease of L^*) after 24 h of irradiation (Table 3, entry 3), suggesting the occurrence of a further polymerization process, probably involving semiquinoid-type free radical species, able to increase the π -conjugation degree of the sample⁸⁶. In the case of sample (VIII) only a modest ΔE^* variation was observed after 24 h, encompassing the decrease of L^* (darkening), the increase of a^* (reddish trend), and the decrease of b^* (yellowish trend) (Table 3, entry 8).

Table 3.

Variations of the chromatic coordinates L^* , a^* , b^* and total color change (ΔE^*) of compound (V) and (VIII).

Entry	Compounds	Treatment	$L^{*,a}$	a^*	b^*	ΔL^*	Δa^*	Δb^*	$\Delta E^{*,b}$
1	V	none	27.2	2.89	8.69				
2	V	UV 2h	29.5	3.04	9.15	2.26	0.15	0.47	2.31
3	V	UV 24h	21.1	4.00	10.0	-6.11	1.11	1.31	6.35
4	V	NaOH 1M 2h	12.1	0.89	2.08	-15.1	-1.99	-6.61	16.6
5	V	NaOH 1M 24h	18.7	1.67	4.04	-8.5	-1.22	-4.65	9.77
6	VIII	none	65.08	5.98	25.59				
7	VIII	UV 2h	62.78	9.12	23.85	-2.30	3.14	-1.74	4.26
8	VIII	UV 24h	61.41	9.79	22.78	-3.67	3.81	-2.81	5.99

^a L^* describes the lightness while a^* and b^* describe the chromatic coordinates on the green-red and blue yellow axes, respectively. ^b ΔE^* is defined as reported in equation (1).

The treatment of the sample (V) with NaOH as selected example produced a great variation of color after 2 h, mainly due to the change of L^* value, in association with a slight decrease of b^* (Table 3, entry 4). The resulting color was darker than the reference, and completely black having lost the b^* contribute. The reflectance spectrum further supports this assessment (Figure 12). Note

that, a lower value of ΔE^* was observed after 24h of treatment (Table 3, entry 5). This reaction pattern can be explained assuming that NaOH can initially favor the complete polymerization of melanin quinones intermediates by a general alkaline catalysis⁸⁷, followed by degradative pathway of lignin for longer reaction time⁸⁸.

Conclusions

We have described the preparation of unique and sustainable bio-ink ingredients functionalized lignin nanocapsules. The synthesis of the pigment was catalyzed by LbL immobilized tyrosinase and laccase, the lignin scaffold performing as vehicle and binder of the pigment. L-DOPA, L-tyrosine, epicatechin and other phenol derivatives were used as reactants to afford a large panel of pigments. When tested together, mixture of phenols afforded different colors, the specific shade being dependent from both the reaction time and the ratio between the two reactants. Pigments were found to be stably linked to lignin, suggesting the formation of covalent bonds between the pigment and the support. The presence of the catechol or hydroquinone moiety in the substrate was essential for the stability of the ink. Oxidative coupling processes occurring through reactive quinone intermediates were probably responsible for this effect. The pigmentation process does not modify the spherical shape of lignin nanocapsules, and the pigment was mainly deposited on the surface of lignin, probably in the proximity of immobilized enzymes. Black bio-inks characterized by low values of reflectance (samples **V**, **XIa** and **XIb**) were obtained. The lowest value of lightness (L^*) was measured for compound (**V**), being so the blackest ink. Two shades of grey were also obtained (samples **VI** and **VII**). Yellow shades were observed with compounds (**VIII**), (**IXb**) and (**Xa-b**). Lastly, compound (**IXa**) showed a light grey-orange color with low saturation, being positioned close to the center of a^*b^*

chromatic diagram. Compounds (V) and (VIII) were resistant to ultraviolet photo-irradiation. In the case of (V) the color darkness increased during the exposition time. Exposure to ultraviolet radiation usually induces the modification of melanin ⁸⁹, suggesting the occurrence of a protective effect, probably due to the known UV shielding capacity of lignin at the nanoscale level ³⁹. Compound (V) was also resistant to alkaline treatment, retaining a black coloration darker than the reference even for prolonged reaction time. The very fact that the pigment is confined on the surface of lignin nanocapsules, which can contemporary act as vehicle, additive and binder for the pigment, encourages to further evaluate the possibility to use this procedure for the preparation of unique polyvalent bio-inks.

Supporting Information.

The following files are available: **Figure S1:** SEM image of compound (I); **Figure S2:** SEM images of compounds (III) and (IV), respectively; **Figure S3:** Melanin concentration after the work-up in KOH solution; **Figure S4:** SEM images of compounds (VI) and (VII), respectively; **Figure S5:** TEM images of compounds (Xa) and compounds (XI), respectively; **Figure S6:** ATR-FTIR analyses of compounds (Xa) and (XIa), respectively.

REFERENCES

- (1) Robert, T. in Green ink in all colors'—printing ink from renewable resources. *Prog. Org. Coat.* **2015**, 78, 287–292.
- (2) Leach, R. H. *The printing ink manual*, **2007** 5th edn. Springer, Dordrecht.
- (3) Kipphan, H. *Handbuch der Printmedien*, **2000** Springer Verlag, Heidelberg.

- (4) Park, J. M.; Kim, Y. H.; Kim, S. B. Development of Solvent-Free Offset Ink Using Vegetable Oil Esters and High Molecular Weight Resin. *J. Oleo Sci.* **2013**, *62*, 345–352.
- (5) Roy, A.S.; Bhattacharjee M.; Mondal R.; Ghosh, S. Development of Mineral Oil Free Offset Printing Ink Using Vegetable Oil Esters. *J. Oleo Sci.* **2007**, *56*, 623–628.
- (6) Karlberg, A.T. in *Colophony: Rosin in Unmodified and Modified Form. In Occupational Dermatology*, **2012** Springer, Berlin, Heidelberg.
- (7) Erhan, S.Z.; Bagby, M.O. Vegetable Oil-based Printing Ink, **1992**, US05122188.
- (8) Mello, V.M.; Oliveira, G.V.; Suarez, P.A.Z. J. Turning used frying oil into a new raw material to printing inks. *Braz. Chem. Soc.* **2013**, *24*, 314–319.
- (9) Bhattacharjee, M.; Roy, A.S.; Ghosh S.; Dey, M. Development of Karanja Oil Based Offset Printing Ink in Comparison with Linseed Oil *J. Oleo Sci.* **2011**, *60*,19–24.
- (10) Ha, Y.B.; Jin, M.Y.; Oh, S.S.; Ryu, D.H. Synthesis of an Environmentally Friendly Phenol-Free Resin for Printing Ink *Bull. Korean Chem. Soc.* **2012**, *33*, 3413–3416.
- (11) Shelton, A.M.C.; Posey-Dowty, J.D.; Rios Perdomo, L.G; Dixon Jr., D.W.; Lucas, P.L.; Wilson, A.K.; Walker, K.R.; Lawniczak, J.E.; Foulk, R.G.; Phan, H.D.; Freeman Jr., C.C. Low Molecular Weight Cellulose Mixed Esters and their Use as Low Viscosity Binders and Modifiers in Coating Compositions, Eastman Chemical Co, **2011**, US2011/0282049A1.
- (12) De Meester, A.S.; Callewaert, C.; De Mol, E.; Van Langenhove, H.; Dewulf,J. The resource footprint of biobased products: a key issue in the sustainable development of biorefineries Bio-fuels Bioprod. *Biorefin. Biof. Pr.* **2011**, *5*, 570–580.

- (13) Ragauskas, A. J.; Beckham, G. T.; Biddy, M. J.; Chandra, R.; Chen, F.; Davis, M. F.; Davison, B. H. Lignin Valorization: Improving Lignin Processing in the Biorefinery. *Science*. **2014**, *344*, 1246843–1246843
- (14) Calvo-Flores, F.G.; Dobado, J.A. Lignin as Renewable Raw Material. *Chem.Sus.Chem.* **2010**, *3*, 1227–1235.
- (15) Kaur, R.; Uppal, S. K. Structural characterization and antioxidant activity of lignin from sugarcane bagasse. *Coll. Polym. Sc.* **2015**, *460*, 2585–2592.
- (16) Azadfar, M.; Gao, A. H.; Bule, M. V.; Chen, S. Structural characterization of lignin: A potential source of antioxidants guaiacol and 4-vinylguaiacol. *Int. J. Biol. Macromol.* **2015**, *75*, 58–66.
- (17) Pan, X.; Kadla, J. F.; Ehara, K.; Gilkes, N.; Saddler, J. N. Organosolv Ethanol Lignin from Hybrid Poplar as a Radical Scavenger: Relationship between Lignin Structure, Extraction Conditions, and Antioxidant Activity. *J. Agric. Food Chem.* **2006**, *54*, 5806–5813.
- (18) Ugartondo, V.; Mitjans, M.; Vinardell, M. P. Comparative antioxidant and cytotoxic effects of lignins from different sources. *Bioresour. Technol.* **2008**, *99*, 6683–6687
- (19) Zimniewska, M.; Batog, J.; Bogacz, E.; Romanowska, B. Functionalization of natural fibres textiles by improvement of nanoparticles fixation on their surface. *J. Fiber Bioeng. Inf.* **2012**, *5*, 321–339.
- (20) Glasser, W.G. *Classification of Lignin According to Chemical and Molecular Structure*. **1999** (Lignin: Historical, Biological, and Materials Perspectives) pp. 216–238.

- (21) Qian, Y.; Qiu, X.; Zhu, S. Lignin: a nature-inspired sun blocker for broad-spectrum sunscreens. *Green Chem.* **2015**, *17*, 320–324.
- (22) Ding, J.; Gu, L.; Dong, W.; Yu, H. Epoxidation Modification of Renewable Lignin to Improve the Corrosion Performance of Epoxy Coating. *Int. J. Electrochem. Sci.* **2016**, *11*, 6256 – 6265.
- (23) Hussin, M.H.; Shah, A.M.; Rahim, A.A.; Ibrahim, M.N.M.; Perrin, D.; Brosse, N. Antioxidant and anticorrosive properties of oil palm frond lignins extracted with different techniques. *Ann. Forest Sci.* **2014**, *72*, 17–26
- (24) Milczarek, G.; Nowicki, M. Carbon nanotubes/kraft lignin composite: Characterization and charge storage properties. *Mat Res. Bull.* **2013**, *48*, 4032-4038.
- (25) Gnedenkov, S. V.; Sinebryukhov, S. L.; Opra, D. P.; Zemnukhova, L. A.; Tsvetnikov, A. K.; Minaev, A. N.; Sergienko, V. I. Electrochemistry of Klason Lignin. *Procedia Chemistry* **2014**, *11*, 96–100.
- (26) Espinoza-Acosta, J. L.; Torres-Chávez, P. I.; Olmedo-Martínez, J. L.; Vega-Rios, A.; Flores-Gallardo, S.; Zaragoza-Contreras, E. A. Lignin in storage and renewable energy applications: A review. *J. Energy Chem.* **2018**, *27*, 1422-1438
- (27) Schilling, P. Lignin Amine Salt-Based Binders for Water-Based Black Ink Formulations, Westvaco Co, **1993**, US05188665.
- (28) Belgacem, M. N., Blayo, A., Gandini, A. Organosolv lignin as a filler in inks, varnishes and paints. *Ind. Crops Prod.* **2003**, *18*, 145–153.
- (29) Finch, C. A. in *Advanced wood adhesives technology*. **1994** (A. Pizzi, Ed.), Chap. V. Dekker, New York.

- (30) Caes, B.; Rivas, M.; Noiro, P.A. Printing Ink with Nitrates Lignin Ester As binder component, **2015** WO 2015/062910 A1.
- (31) Michael, D. Matzinger Water-based ink jet ink compositions containing carboxylated lignin **1999**, Grant US6045606A Westvaco Corporation.
- (32) Miller, J.E.; Jr.Dilling, P. Water-based printing ink compositions containing a lignin acetate binder, **1985** US Grant US4612051A Westvaco Corp.
- (33) Beisl, S.; Friedl, A.; Miltner, A. Lignin from Micro- to Nanosize: Applications. *Int. J. Mol. Sci.* **2017**, *18*, 2367.
- (34) Figueiredo, P.; Lintinen, K.; Kiriazis, A.; Hynninen, H.; Liu, Z.; Bauleth-Ramos, T.; Rahikkala, A.; Correia, A.; Kohout, T.; Sarmiento, B.; Yli-Kauhaluoma, J.; Hirvonen, J.; Ikkala, O.; Kostiaainen, M. A.; Santos, H. A. In vitro evaluation of biodegradable lignin-based nanoparticles for drug delivery and enhanced antiproliferation effect in cancer cells. *Biomaterials* **2017**, *121*, 97-108.
- (35) Tian, D.; Hu, J.; Bao, J.; Chandra, R. P.; Saddler, J. N.; Lu, C. Lignin valorization: lignin nanoparticles as high-value bio-additive for multifunctional nanocomposites. *Biotech. Biofuels* **2017**, *10*, 192].
- (36) Vukoje, M.; Miljanić, S.; Hrenović, J.; Rožić, M. Thermochromic ink–paper interactions and their role in biodegradation of UV curable prints. *Cellulose*, **2017**, *25*, 6121–6138.
- (37) Schilling, P. Submicron lignin-based binders for water-based black ink formulations, **1992** Westvaco Corporation US005192361A.

- (38) Moon, R.J.; Martini, A.; Nairn, J.; Simonsen, J.; Youngblood, J. Cellulose nanomaterials review: structure, properties and nanocomposites. *Chem. Soc. Rev.* **2011**, *40*, 3941–3994.
- (39) Piccinino, D.; Capecchi, E.; Botta, L.; Bizzarri, B. M.; Bollella, P.; Antiochia, R.; Saladino, R. . Layer-by-Layer Preparation of Microcapsules and Nanocapsules of Mixed Polyphenols with High Antioxidant and UV-Shielding Properties. *Biomacromol.* **2018**, *19*, 3883–3893.
- (40) Ball, V.; Bernsmann, F.; Betscha, C.; Maechling, C.; Kauffmann, S.; Senger, B.; Voegel J.C.; Schaaf, P.; Benkirane-Jessel N. Polyelectrolyte multilayer films built from poly(L-lysine) and a two-component anionic polysaccharide blend. *Langmuir* **2009**, *25*, 3593-6000.
- (41) Sipponen, M. H.; Farooq, M.; Koivisto, J.; Pellis, A.; Seitsonen, J.; Österberg, M. Spatially confined lignin nanospheres for biocatalytic ester synthesis in aqueous media. *Nature Comm.* **2018**, *9*, Article number: 2300
- (42) Capecchi, E.; Piccinino, D.; Delfino, I.; Bollella, P.; Antiochia, R.; Saladino, R. Functionalized Tyrosinase-Lignin Nanoparticles as Sustainable Catalysts for the Oxidation of Phenols. *Nanomaterials.* **2018**, *8*, 438.
- (43) Frommhagen, M.; Mutte, S.K.; Westphal, A.H.; Koetsier, M.J.; Hinz, S.W.A.; Visser, J.; Kabel, M.A. Boosting LPMO-driven lignocellulose degradation by polyphenol oxidase-activated lignin building blocks. *Biotechnol. Biofuels.* **2017**, *10*, 1.
- (44) Shleev, S.; Jarosz-Wilkolazka, A.; Khalunina, A.; Morozova, O.; Yaropolov, A.; Ruzgas, T.; Gorton, L. Direct electron transfer reactions of laccases from different origins on carbon electrodes. *Bioelectrochem.* **2005**, *67*, 115–124.

- (45) Polak, J.; Jarosz-Wilkolazka, A. Structure/Redox potential relationship of simple organic compounds as potential precursors of dyes for laccase-mediated transformation. *Biotechnol. Prog.* **2011**, *28*, 93–102.
- (46) Lievonen, M.; Valle-Delgado, J. J.; Mattinen, M.-L.; Hult, E.-L.; Lintinen, K.; Kostianen, M. A.; Paananen, A.; Szilvay, G. R.; Setälä, H.; Österberg, M. A simple process for lignin nanoparticle preparation. *Green Chem.* **2016**, *18*, 1416–1422.
- (47) Jiang, C.; He, H.; Jiang, H.; Ma, L.; Jia, D. M. Nano-lignin filled natural rubber composites: Preparation and characterization. *Express Polym. Lett.* **2013**, *7* 480-493.
- (48) Grönqvist; S., Viikari; L.; Niku-Paavola, M. L.; Orlandi, M.; Canevali, C.; Buchert, J. Oxidation of milled wood lignin with laccase, tyrosinase and horseradish peroxidase. *Appl Microbiol. Biotech.* **2005**, *67*, 489-94
- (50) Makpol, S. Comparable down-regulation of TYR, TYRP1 and TYRP2 genes and inhibition of melanogenesis by tyrostat, tocotrienol-rich fraction and tocopherol in human skin melanocytes improves skin pigmentation. *Clin. Ter.* **2014**, *165*, 39-45.
- (51) ISO 11664-4. Colorimetry - Part 4: CIE 1976 L*a*b* Colour space, **2008**.
- (52) Mattinen, M.L.; Valle-Delgado, J.J.; Leskinen, T.; Anttila, T.; Riviere, G.; Sipponen, M.; Österberg, M. Enzymatically and chemically oxidized lignin nanoparticles for biomaterial applications. *Enzyme Microb. Technol.* **2018**, *111*, 48–56.
- (53) Decher, G. Fuzzy Nanoassemblies: Toward Layered Polymeric Multicomposites. *Science*, **1997**, *277*, 1232–1237.

- (54) Piccinino, D.; Delfino, M.; Botta, G.; Crucianelli, M.; Grossi, V.; Passacantando, M.; Saladino, R. Highly efficient synthesis of aldehydes by layer-by-layer multi-walled carbon nanotubes (MWCNTs) laccase mediator systems. *Appl. Catal., A. General* **2015**, *499*, 77-88.
- (55) Glazer, A. N.; Nikaido, H. in *Microbial Biotechnology: fundamentals of applied microbiology*, **1995** pp.340. San Francisco: W. H. Freeman.
- (56) Richert, L.; La Valle P.; Payan, E.; Shu, X.Z.; Prestwich, G.D.; Stultz, J.-F.; Schaaf, P.; Voegel, J.-C.; Picart, C. Layer by Layer Buildup of Polysaccharide Films: Physical Chemistry and Cellular Adhesion Aspects. *Langmuir* **2004**, *20*, 448-458.
- (57) Liu, Y.; Ai, K.; Liu, J.; Deng, M.; He, Y.; Lu, L. Dopamine-melanin colloidal nanospheres: An efficient near-infrared photothermal therapeutic agent for in vivo cancer therapy. *Adv. Mater.* **2013**, *25*, 1353–1359.
- (58) Neifar A.; Ben Adelmalek I.; Bouajila G.; Kolsi R.; Nejmeddine Bradai M.; Abdelmouleh A.; Gargourib A.; Ayed N. Purification and incorporation of the black ink of cuttlefish *Sepia officinalis* in eye cosmetic products. *Color. Technol.* **2013**, *129*, 150–154.
- (59) Ito, S. A chemist's view of melanogenesis. *Pigment Cell Res.* **2003**, *16*, 230–236.
- (60) Reale, S.; Crucianelli, M.; Pezzella, A.; d'Ischia, M.; de Angelis, F. Exploring the frontiers of synthetic eumelanin polymers by high-resolution matrix-assisted laser/desorption ionization mass spectrometry. *J. Mass Spectrom.* **2012**, *47*, 49–53.
- (61) Nofsinger, J. B.; Forest, S. E.; Eibest, L. M.; Gold, K. A.; Simon, J. D. Probing the Building Blocks of Eumelanins Using Scanning Electron Microscopy. *Pigment Cell Res.* **2000**, *13*, 179–184.

- (62) Kim, D. J.; Ju, K. Y.; Lee, J. K. The Synthetic Melanin Nanoparticles Having An Excellent Binding Capacity of Heavy Metal Ions. *Bull. Korean Chem. Soc.* **2012**, *33*, 3788–3792.
- (63) Xiao, M.; Chen, W.; Li, W.; Zhao, J.; Hong, Y-lee; Nishiyama, Y.; Miyoshi, T.; Shawkey M.D.; Dhinojwala, A. 2018 Elucidation of the hierarchical structure of natural eumelanins. *J. R. Soc. Interface.* **2018**, *15*, 2018 00 45.
- (64) Xiong, F.; Han, Y.; Wang, S.; Li, G.; Qin, T.; Chen, Y.; Chu, F. Preparation and Formation Mechanism of Renewable Lignin Hollow Nanospheres with a Single Hole by Self-Assembly. *ACS Sustainable Chem. Eng.* **2017**, *5*, 2273–2281.
- (65) Xing, Q.; Buono, P.; Ruch, D.; Dubois, P.; Wu, L.; Wang, W. J. Biodegradable UV-Blocking Films through Core–Shell Lignin–Melanin Nanoparticles in Poly(butylene adipate-co-terephthalate). *ACS Sustainable Chem. Eng.* **2019**, *7*, 4147–4157.
- (66) Bizzarri, B.M; Martini, A.; Serafini, F.; Aversa, D.; Piccinino, D.; Botta, L.; Berretta N; Guatteo, E.; Saladino, R. Tyrosinase mediated oxidative functionalization in the synthesis of DOPA-derived peptidomimetics with anti-Parkinson activity. *RSC Adv.*, **2017**, *7*, 20502-20509.
- (67) Ilani, A.; Krakover, T. Diffusion- and reaction rate-limited redox processes mediated by quinones through bilayer lipid membranes. *Biophysical J.* **1987**, *51*, 161-167
- (68) Strube, O.I.; Büngeler, A.; Bremser, W. Site-Specific In Situ Synthesis of Eumelanin Nanoparticles by an Enzymatic Autodeposition-Like Process. *Biomacromol.* **2015**, *16*, 1608–1613.47.

- (69) Surwase, S. N.; Patil, S.A.; Jadhav, S.B.; Jadhav, J.P. Optimization of l-DOPA production by *Brevundimonas* sp. SGJ using response surface methodology. *Microb. Biotechnol.* **2012**, *5*, 731–737.
- (70) Al Khatib, M.; Harir, M.; Costa, J.; Baratto, M.; Schiavo, I.; Trabalzini, L.; Pollini, S.; Rossolini, G.; Basosi, R.; Pogni, R. Spectroscopic Characterization of Natural Melanin from a *Streptomyces cyaneofuscatus* Strain and Comparison with Melanin Enzymatically Synthesized by Tyrosinase and Laccase. *Molecules.* **2018**, *23*, 1916.
- (71) Robbins, R. in *Chemical and physical behavior of human hair, in Genetic Control/Involvement in Hair Fiber Traits*, **2012** chapter 3, pp. 177–203, Springer, Berlin, Germany, 5th edition.
- (72) Yaropolov, A. I.; Skorobogat'ko, O.V., Vartanov, S.S., Varfolomeyev, S.D. Laccase. *Appl. Biochem. Biotechnol.* **1994**, *49*, 257–280.
- (73) Riva, S. Laccases: blue enzymes for green chemistry. *Trends Biotech.* **2006**, *24*, 219-226.
- (74) Crestini, C.; Perazzini, R.; Saladino, R. Oxidative functionalisation of lignin by layer-by-layer immobilised laccases and laccase microcapsules. *Appl. Cat. A: General* **2010**, *372*, 115–123
- (75) Jeon, J.R., Kim, E.J., Murugesan, K., Park, H.K., Kim, Y.M., Kwon, J.H., Chang, Y.S. Laccase-catalysed polymeric dye synthesis from plant-derived phenols for potential application in

hair dyeing: Enzymatic colourations driven by homo- or hetero-polymer synthesis. *Microbial Biotech.* **2010**, *3*, 324–335.

(76) Song, J.E.; Su, J.; Noro, J.; Cavaco-Paulo, A.; Silva, C.; Kim, H. R. Bio-coloration of bacterial cellulose assisted by immobilized laccase. *AMB Expr.* **2018**, *8*, 19.

(77) Kim, S.; Moldes, D.; Cavaco-Paulo, A. Laccases for enzymatic colouration of unbleached cotton. *Enzyme Microb. Technol.* **2007** *40*, 1788–1793.

(78) Slagman, S.; Zuilhof, H.; Franssen, M.C.R. Laccase-Mediated Grafting on Biopolymers and Synthetic Polymers: A Critical Review. *ChemBioChem* **2018**, *19*, 288–311.

(79) Maoela, M.S.; Arotiba, O.A.; Baker, P.G.; Mabusela, W.T.; Jahed, N.; Songa, E.A.; Iwuoha, E.I. Electroanalytical determination of catechin flavonoid in ethyl acetate extracts of medicinal plants. *Int. J. Electrochem. Sci.* **2009**, *4*, 1497-1510.

(80) Lide, D.R.; Milne G.W.A. in Handbook of Data on Organic Compounds. Volume I. 3rd ed. CRC Press, Inc. Boca Raton,FL. **1994.**, p. V2: 1348.

(81) Yang, J.; Cohen Stuart, M.A.; Kamperman, M. ChemInform Abstract: Jack of All Trades: Versatile Catechol Crosslinking Mechanisms. *Chem. Soc. Rev.*, **2014**, *43*, 8271-8298.

- (82) Nyanhongo, G.S., Kudanga, T., Nugroho Prasetyo, E., Gübitz, G.M. Enzymatic Polymer Functionalisation: Advances in Laccase and Peroxidase Derived Lignocellulose Functional Polymers. *Adv. Biochem. Engin./Biotechnol.* **2010**, *125*, 47–68.
- (83) Ajao, O.; Jeaidi, J.; Benali, M.; Restrepo, A.M.; El Mehdi, N.; Boumghar, Y. Quantification and Variability Analysis of Lignin Optical Properties for Colour-Dependent Industrial Applications. *Molecules*, **2018**, *23*, 1–21.
- (84) Aydemir, C.; Yenidoğan, S. Light fastness of printing inks: A review. *Journal of Graphic Engineering and Design*. **2018**, *9*, 37–43.
- (85) Mokrzycki, W.S.; Tatol, M. Colour difference ΔE -A survey. *Machine Graphics and Vision*. **2011**, *20*, 383–411.
- (86) Solano, F. Melanins: Skin Pigments and Much More—Types, Structural Models, Biological Functions, and Formation Routes. *New Journal of Science*. **2014**, 1–28.
- (87) Ragimov, A. V.; Mamedov, B. V.; Liogonkii, B. The alkali initiated polymerization of p-benzoquinone. *Polymer Science USSR* **1977**, *19*, 2922–2928
- (88) Erdocia, X.; Prado, R.; Corcuera, M.Á.; Labidi, J. Base catalyzed depolymerization of lignin: Influence of organosolv lignin nature. *Biomass and Bioenergy*. **2014**, *66*, 379–386.

(89) Ito, S.; Wakamatsu, K.; Sarna, T. Photodegradation of Eumelanin and Pheomelanin and Its Pathophysiological Implications. *Photochem. and Photobiol.* **2017**, *94*, 409–420.

TOC/Abstract Graphic.

SYNOPSIS. Functionalized lignin nanoparticles catalyze pigmentation reaction in the presence of phenol derivatives to yield a unique polyvalent bio-ink.