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Elucidating the chemical nature of laccase-modified alkyl gallates

Oriol Cusola[1], Cristina Valls[1], Teresa Vidal[1], M. Blanca Roncero[1]*

[1]*CELBIOTECH_Paper Engineering Research Group. Universitat Politècnica de Catalunya-BarcelonaTech. Colom 11, E-08222 Terrassa, Spain*

*Corresponding author. Tel.: +34 93 739 82 10; Fax.: +34 93 739 81 01. E-mail address: blanca.roncero@upc.edu (M. Blanca Roncero)

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In the first part of the present work, relevant findings concerning the laccase (Lacc) activation of alkyl gallates, and its implementation in developing hydrophobic properties on cellulose-based substrates are reported. Surface state energy by water contact angle (CA), absorption tests, and $Cobb_{60}$ measurements were used to assess the hydrophobic behavior of treated substrates. SEM images of paper samples treated with functionalization solutions (FS) revealed the presence of lauryl gallate (LG, dodecyl 3,4,5,-trihydroxybenzoate) particles attached to fibre surfaces. Secondly, the chemical structure of the enzyme-oxidized LG and several gallates of variable chain length was elucidated by using Fourier transform infrared (FTIR) spectroscopy, and a plausible oxidation mechanism was developed. Based on them, the hydrocarbon chain of LG remains unaltered after enzyme oxidation, while the aromatic ring is significantly altered to form acidic adducts undergoing strong hydrogen bonding. Possible routes for the grafting of enzyme-modified LG to cellulose are proposed.

Keywords: Laccase; cellulose; alkyl gallates; hydrophobicity; FTIR

Introduction

Gallic acid and its alkyl esters constitute an important group of biogenic molecules possessing a wide spectrum of interesting properties such as antibacterial [1,2] antioxidant [3–5] antifungal [6], hydrophobic [7] and antitumoral action [8–10]. Some gallic acid derivatives such as methyl, propyl, octyl and dodecyl gallates are widely used in food manufacturing, as well as in the pharmaceutical and cosmetic industries. Most of the properties shown by alkyl gallates are required specifications in materials for packaging or medical uses. Therefore, using alkyl gallates in the production of such materials is indeed an interesting alternative. A number of studies have reported on the use of gallate derivatives to enhance the properties of composites and films, and to provide sustained release of antioxidant during storage [11–13].

On the other side, the interest in using biotechnological methods to obtain materials with improved properties has also grown substantially [14–17]. Using enzymes to produce advanced materials has some advantages over chemical methods, allowing to operate under milder conditions, high specificities, efficiency, easy control, and environmental friendliness [18,19]. Laccases (Lacc, EC 1.10.3.2) are blue copper oxidases capable of oxidizing a variety of aromatic compounds by using oxygen as electron acceptor and giving water as by-product.

Laccases provide an eco-friendly approach to improve cellulose properties by means of enzyme-assisted grafting. Their ability to graft low molecular weight phenolic compounds onto cellulose has been demonstrated by several authors [20–23]. Specifically, the laccase-alkyl gallate system was used to confer new properties to composites and cellulose-based materials. For example, Filgueira et al., [24] studied laccase-assisted grafting of octyl gallate and lauryl gallate onto thermomechanical fibers, increasing their compatibility with polymer matrixes in order to achieve bio-based 3D printing filaments. Similarly, alkyl gallates with different aliphatic chain length were laccase-grafted onto jute fabric to increase its hydrophobicity and to maximize compatibility in jute/polypropylene composites [16,25,26]. Gaffar et al., [27] studied the laccase-catalysed grafting of various gallates onto wool to obtain multifunctional textile materials with antioxidant, antibacterial and water-repellent properties. Finally, lauryl gallate was used to hydrophobize cellulose fibres [28] and nanofibrillar cellulose [29]. Some authors have inferred the grafting mechanisms, demonstrating that enzyme-activated phenolics stick cellulose through cellulose-bound lignin [30–33]. This requires laccase-assisted activation of fiber surfaces followed by radical coupling.

All the enzymatic treatments listed above were conducted under the form of aqueous suspensions. This means that fibers, enzyme, and alkyl gallate compounds were present simultaneously in the reaction medium until the end of the treatment, resulting in enzyme-modified fibers. These modified fibers might then be utilized to manufacture products like paper, fabrics or composites, which are expected to show the new functionalities. However, applying treatments on fibrous suspensions may entail several drawbacks, e.g. possible interferences in the interfibre bonding, higher consumption of compounds, or limitations in terms of working conditions (pH, temperature, consistency and reaction time).

A method to circumvent these disadvantages is the two-step process developed in previous works [34–37]. In this new approach, the desired functionalities are achieved upon treatment of finished substrates with a functionalization solution (FS), which is prepared without the presence of any cellulosic material during the reaction. Significant advantages are drawn from this new approach, e.g. it does not affect the binding capacity of the fibers neither the substrate-forming process, it uses reduced amounts of compounds, it has higher effectivity since compounds stay on the surface of the substrates, and it is versatile in terms of the application methods, just to list a few. There is, however, little knowledge about the nature of the chemical changes that laccases endow to alkyl gallates, or the mechanisms by which these enzyme-modified molecules bond to cellulose.

In the present work, these chemical changes are investigated and possible mechanisms by which these molecules graft to cellulose, proposed. First, several data on the ability of enzyme-modified gallates to confer hydrophobic behavior to cellulose-based materials are presented. Commercial and eucalyptus paper sheets were treated with enzyme-modified LG and other gallates and their hydrophobic behavior was assessed by means of CA, absorption and SEM. Finally, the FTIR findings about the chemical changes undergone by alkyl gallates upon enzymatic reaction are reported. The results allowed establishing various potential mechanisms for the LG-cellulose binding.

Materials & methods

Frequent abbreviations

CA: Contact angle

Lacc: Laccase

LG: Lauryl gallate, dodecyl 3,4,5-trihydroxybenzoate

SL: Sulphonated lignin or lignosulfonates

FS: Functionalization solution containing Lacc, LG, and SL

FTIR: Fourier-transform infrared

GA: Gallic acid, 3,4,5-trihydroxybenzoic acid

MG: Methyl gallate, methyl 3,4,5-trihydroxybenzoate

EG: Ethyl gallate, ethyl 3,4,5-trihydroxybenzoate

PG: Propyl gallate, propyl 3,4,5-trihydroxybenzoate

BG: Butyl gallate, butyl 3,4,5-trihydroxybenzoate

OG: Octyl gallate, octyl 3,4,5-trihydroxybenzoate

SG: Stearyl gallate, octadecyl 3,4,5-trihydroxybenzoate

Enzyme and chemicals

The enzyme used was laccase (Lacc) from Trametes villosa supplied by Novozymes® (Bagsvaerd, Denmark). Gallic acid (GA; 170.12 g/mol), methyl gallate (MG; 184.15 g/mol), ethyl gallate (EG; 198.17 g/mol), propyl gallate (PG; 212.2 g/mol), butyl gallate (BG; 226.23 g/mol), octyl gallate (OG; 282.33 g/mol), lauryl gallate (LG; 338.44 g/mol) and stearyl gallate (SG; 422.606 g/mol) were purchased from Sigma–Aldrich®. Soluble sulphonated kraft lignin (SL) was obtained from Borreegard® and used as received.

Preparation of the functionalization solutions (FS)

The enzymatic treatments were performed in an Ahiba Spectradye dyeing apparatus from Datacolor® equipped with closed vessels 250 mL in volume with final concentrations equivalent to 50 mM sodium tartrate buffer (pH 4), gallate compound (1.2 g/L), lignosulfonate (SL) (1.2 g/L), and 1.2 U/mL laccase. The molar concentration of each gallate compound was: GA, 7.1 mM; MG, 6.5 mM; EG, 6.1 mM; PG, 5.7 mM; BG, 5.3 mM; OG, 4.3 mM; LG, 3.5 mM; and SG, 2.8 mM). SL was introduced in the reaction to improve dispersion of the gallate compound. If the gallalte was hydrophobic and no-solubilisation in water was produced, then it was applied as a colloidal suspension prepared upon ultrasonication, achieving a reduction in the effective size and ensuring homogeneous dispersion. Following, the enzyme was added and the beakers were kept stirred during 4 h reaction. The reaction was stopped by quenching the beakers with cold water. Several control treatments were also performed to elucidate the role of each of the compounds present in the FS.

Paper treatment with the FS

Eucalyptus ECF pulp was supplied by ENCE® (Spain) and used to prepare handsheets on a Rapid-Köten lab former according to ISO 5269-2:2004. Uncoated 67 g/m^2 paper was obtained from Torraspapel®. The obtained solutions (FS), were applied at room temperature to the paper sheets by impregnation/immersion. With this purpose, the paper sheets were cut into circular pieces 4 cm in diameter and soaked in the FS by keeping the sheet submerged for 3 seconds. Then the paper sheet was removed, spread onto blotting paper, and allowed to dry in a normalized atmosphere (23°C, 50% RH).

Assessment of the hydrophobic behaviour

The water contact angle (CA) was measured by using a Dataphysics OCA15EC contact angle goniophotometer using an image capture ratio of 25 frames/s. A 4 µL water drop was delivered to the sample surface. At least 10 measurements were made for each liquid probe. Also, changes in contact angle were monitored until complete absorption of each water drop. $Cobb_{60}$ absorption tests were performed according to the international standard ISO 535:199.

SEM analysis

Scanning electron microscopy (SEM) was used to examine the surface of paper sheets treated with the functionalization solution (FS) and control solutions. Micrographs were obtained with a JEOL JSM-6400 microscope operating at 5–10 kV under moderate vacuum. Specimens were previously cut into small pieces and coated with a very thin (12 nm) Au–Pd layer on an SCD005 sputter coater to obtain a conductive surface. The particle size of the enzyme-modified LG observed on the paper surface was measured from SEM images using ImageJ software (see supporting information file).

FTIR analysis

For the FTIR analysis of the enzyme-modified LG and other gallates, 200 mL of the FS was prepared using the doses and reaction conditions explained previously. However, SL was not introduced in the reaction in order to avoid disturbances coming from this compound in the FTIR analysis. Then, the solutions were centrifuged at 6000 rpm during 90 min, and the supernatant eliminated from the sample. Afterwards, the sample was allowed to dry at room temperature until a dry solid residue was obtained, and the solid was placed into a desiccator overnight. Finally, it was finely grinded and mixed with potassium bromide for KBr pellets preparation. Samples were measured using a Nicolet 6700 FT-IR Spectrometer equipped with spectrum software in the range of 4000–400 cm-1.

In the ATR-FTIR experiments with increasing-length gallates, the solid residue was directly analyzed on a Perkin Elmer Spectrum 100 FT-IR spectrometer equipped with universal ATR sampling accessory by performing 32 scans at 1 cm–1 intervals over the wavenumber range 4000–550 cm–1. Pure gallates (i.e. gallates subject to no enzymatic modification) were also analysed. The solid residues were placed directly on the ATR accessory, and the measuring cell was washed with de-ionized water and ethanol between measurements.

Results and discussion

In the first part of this work, previous and current efforts on the use of LG and other alkyl gallates to achieve the enzymatic functionalization of cellulosic substrates are outlined. In the second part, FTIR studies undertaken to elucidate the chemical nature of enzyme-modified alkyl gallates, with a particular emphasis on LG compound are reported.

Enzymatic modification of alkyl gallates and substrate treatments

Laccases require aqueous medium for its action. However, LG compound is highly hydrophobic, and tends to aggregate (i.e., accumulate and clump together).

Therefore, LG dispersion was a key aspect to optimize the action of the enzyme. Thus, sulfonated lignins (SL) were added, significantly increasing dispersion, among other benefits [34,36]. The best performance was observed when reaction (Laccase-LG), was conducted under the presence of SL, resulting in a three-compound reaction mixture named functionalization solution (FS). This resulting product (FS) was able to increase the hydrophobic behaviour of cellulose-based materials after impregnation and drying (Figure 1a). As shown in Figure 1b, the water contact angle and $Cobb_{60}$ values significantly increased upon treatment with FS. The treatment with FS is able not only to modify the surface state energy, but also the water absorption of the material, as depicted in Figure 1c.

The Lacc, LG and SL dose and reaction time to achieve the FS were selected according to previous studies. Further experiences to assess the influence of LG and SL dosing (see Table S1 and Figure S1 in the supporting information file) were performed. The results showed that short reaction times (between 1 and 2 hours) were not effective in developing the hydrophobic property, either by using low or high doses. Possibly, enzyme was not able to complete oxidation in the time interval considered. For long reaction periods (between 2 and 4 hours), the use of lower concentrations seemed to be more effective in the development of the hydrophobic property. The highest hydrophobic level was attained when using 1.2 g/L for LG and SL and 1.2 U/mL of enzyme, with a reaction time of 3 h. The use of very high doses (x20) with either long or short reaction time is deleterious as to enzymatic modification.

The effect of the alkyl chain length was also investigated. As shown in Figure 1d, an increased hydrophobic behaviour was observed after treatment with functionalization solutions prepared with increasing-chain-length gallates. However, very long gallates (longer than LG), namely Stearyl Gallate, (with 18 carbons in the chain), are deleterious as far as the hydrophobic property. The reason was the impossibility to disperse this compound in aqueous medium, due to its very high hydrophobic nature and very low solubility.

SEM analysis of cellulosic material treated with FS

The surface of cellulose and FS-treated cellulose specimens was examined by scanning electron microscopy (SEM, Figure 2). Figures 2a and 2b show the surface of non-treated cellulose, where smooth and clean fibres are appreciated. By contrast, treatment with FS (Figures 2c and 2d) caused significant morphological changes in the fibres. Thus, after the treatment with FS, small nanosized particles (90 to 600 nm) appeared on the surface (See Figure S3 in supporting information file). Such particles consisted of enzyme-modified LG, and contributed to the hydrophobic behaviour of the paper sheets. The particles are barely distinguishable in the macroscopic image of FS-treated paper (Figure 2c) —only a few of the larger ones can clearly be seen—, but apparent in the magnified view of the surface in Figure 2d. Worth special note is the wide range of LG particle sizes present in the image (aggregates from 100 to more than 500 nm in size), and so is the fact that gold-sputtering the fibre surfaces —a requirement for SEM analysis of non-conductive materials such as cellulose— coated the sample with a thin (10–30 nm) layer of gold, which may have completely covered small LG particles and hidden them from view.

Paper sheets were also impregnated with several control treatments to elucidate the effect of each individual compound in the FS, and combinations thereof (Figure S2). Only the treatments in which LG was present caused deposition of LG particles onto fibre surfaces. Although LG and LG+Lacc treatments produced similar effect (as far as the SEM images), a significant difference in terms of attachment of LG particles was apparent. Thus, the specimen treated with the LG control consisted of LG-deposited particles whereas that treated with Lacc+LG contained firmly attached particles on the fibre surface, consistent with the previously found results [37]. Unlike the surface particles of the Lacc+LG treatment, those resulting from the LG treatment were easily removed by washing with water. The effects of the enzyme were thus quite clear: altering the chemical nature of LG and facilitating its anchoring onto fibre surfaces.

FTIR analysis of pure and enzyme-modified LG

Figure 3 shows the FTIR absorbance spectra for LG before and after oxidation by the enzyme (LG+Lacc). The overall shape of the spectrum for LG changed upon enzymatic modification; thus, the initial LG spectrum contained a number of strong, well-defined peaks, whereas in that for enzyme-treated LG, some peaks disappeared or shifted wavenumber.

The peaks at 2848 and 2916 cm^{-1} corresponded to methylene and methyl groups in the pure LG hydrocarbon chain. As shown, these peaks remained unchanged upon enzymatic modification of LG, indicating that enzyme oxidation probably did not alter the hydrocarbon chain. The adsorption band at 1668 cm^{-1} for unmodified LG must correspond to C=O stretching in the ester —which usually appears at higher wavenumbers in esters. The reason for this shift may be due to the connection of the ester with the aromatic ring (an aryl ester) [38], and also to hydrogen bonding interactions of the ester with hydroxyl groups in the same molecule (intra-molecular interactions) or in others (inter-molecular interactions) [39] (See Figure S4 in supporting information file). Intra-molecular interactions should be independent of the gallate chain length; in fact, the C=O stretching band for the ester group in other gallates appeared at the expected frequencies, as it will be discussed in the next sections. A longer chain may thus prevent inter-molecular interactions but promote intra-molecular interactions.

The carbonyl peak appeared at higher frequencies in the spectrum for enzyme-modified LG (1718 cm^{-1}). Since the action of the enzyme focuses on the phenol [40], the shift may be a result of some alteration of the aromatic ring such as cleavage and the formation of a carboxylic acids [41], whose peaks usually appear in a very specific frequency range (1725–1700 cm^{-1}). The ester peak did not disappear and, probably, the ester and carboxylic acid peaks merged at 1718 cm^{-1}. Further evidence of ring opening/alteration or also quinone formation was obtained from the LG peaks observed in the 1600-1450 cm^{-1} range, which correspond to C=C–C aromatic ring bonds. Such peaks were considerably different in the enzyme-modified LG spectrum (specifically, the peak at 1533 nm disappeared altogether).

Stretching vibrations of the hydroxyl group in the unmodified LG molecule reflected in a broad band in the range 3600–3300 cm^{-1} containing peaks at 3450–3348 cm^{-1}. There were no strong peaks for unbounded hydroxyl groups in the range 3645–3600 cm^{-1}; rather, the broad band at 3600–3300 cm^{-1} is suggestive of H-bonding of hydroxyl groups in the same (intra-molecular hydrogen bonding) or neighboring molecules (inter-molecular bonding). The reduction and further broadening of this band observed in enzyme-modified LG was also an indication of the disappearance of hydroxyl groups and formation of carboxyl groups; a highly characteristic large shift to lower frequencies by effect of carboxyl groups establishing extremely strong hydrogen bonds was observed.

The reaction mechanism for the oxidation of gallic acid proposed by Tulyathan et al., [42], in combination with our FTIR results, can be used to understand the nature of the LG oxidation mechanism caused by laccase. Laccases exhibit specificity for phenols, diphenols, aryldiamines, aminophenols, benzenethiols and hydroxyindoles; this was confirmed by FTIR, which exposed major changes in the wavenumbers corresponding to the aromatic ring but none in those for the hydrocarbon chain. Also, the hypothesis is based on the reported bacterial degradation pathway for phenylacetate, which represents an example of cleavage of an aromatic compound while retaining an ester bond [43–45]. The LG oxidation mechanism proposed here involves the following steps (Figure 4): (1) Laccase catalyses the oxidation of LG by removing electrons and hydrogen ions from phenolic hydroxyl groups to produce phenoxy radicals [40] (2) which are further oxidized to quinones (3), thereby causing cleavage of the aromatic ring and producing hydrophilic carboxyl groups in the form of muconic acid derivatives [46,47] (4). The next step is pictured as alternative pathways that account for condensation of hydroxyl groups in the alcohol and carboxyl groups (–COOH) to form a lactone [48] (5) or decarboxylation and further oxidation (6, 7). According to some authors, laccase–mediator systems can form activated oxygen species such as hydroxyl radical ($HO^{\bullet}$) and superoxide anion radical ($O2^{\bullet-}$) [49]. Such radicals can also boost oxidation of the LG product by breaking C–C bonds and causing cleavage of aromatic rings and scission of conjugated double bonds as a result [50].

Formation of stable dimers by regeneration of hydroquinones (after step 3) might be difficult due to steric hindrances caused by the long hydrocarbon chain of LG. As proposed by Tulyathan et al., [42] for the oxidation of gallic acid, the reaction must lie between the formation of single-molecule open-ring acidic products or oxidation of a dimer to acidic products. We hypothesize that the laccase reaction produces single LG molecules with open-ring acid moieties anchored to the alkyl chain via the original ester (Figure 4). The strong anionic character of such molecules seems to be the main agent enhancing retention on cationic surfaces.

The dark color of the Lacc–LG reaction products is ascribed to the formation of quinone structures as in step (3) of Figure 4. However, such color decreased as the reaction developed, suggesting that further oxidation increased the likelihood of obtaining larger amounts of the colorless molecule formed in step (7) of Figure 4, and hence lighter colored reaction product.

The formation of polymerized species of LG, such as dimers or trimers, might be limited by the above-described potential steric hindrances, and confirmed by the decreased turbidity, as observed in previous works. Dynamic light scattering and turbidity measurements showed LG particle size to be decreased by effect of the enzyme (viz. by its reducing the turbidity of the reaction product as assessed with a Turbiscan®) [37]; such an effect was present after long treatment —as long as some enzymatic activity remained. These results suggested clarification of the solution by effect of particle size reduction and quinone destruction, and formation of none of the above-mentioned condensation adducts.

Finally, as confirmed by FTIR data, the resulting species Step (7) in Figure 4 can establish strong hydrogen bonds through the carboxyl groups formed (See Figure S4 in supporting information file).

FTIR-ATR analysis of gallates with increasing chain length and the effect of the enzymatic modification

The FTIR study was extended to other compounds of the gallate family in order to strengthen our understanding of the relationship between the gallates’ structure and their chemical enzymatic changes. Each gallate was laccase-oxidized under the reaction conditions described, albeit in the absence of SL in order to avoid disturbances coming from the presence of this compound (as explained in the materials & methods section) The obtained products were concentrated and dried. The resulting powders consisting of enzymatically-modified gallates, and pure gallates as well, were assessed by FTIR-ATR. Figure 5 shows the FTIR spectra for the pure (Figure 5a) and enzyme-modified gallates (Figure 5b) over the wavenumber range 3600–2600 cm^{-1}; Figures 5c and 5d show those in the spectral region from 1750 to 600 cm^{-1}.

Based on Figures 5a and 5b, the hydrocarbon chains remained intact after reaction with the enzyme; the peaks for methyl (–CH3, 2850 cm^{-1}) and methylene groups (–CH2, 2916 cm^{-1}) remained unchanged. This was particularly so for the long-chain gallates and, especially, for PG and longer gallates. The enzyme caused no chemical changes in the alkyl chain. The peaks for –CH2 and –CH3 groups in the hydrocarbon chain increased intensity with increasing chain length; this was especially so for methylene groups, by effect of their increase in number with increasing chain length.

The broad band from 3570 to 3200 cm^{-1} for the pure gallates (Figure 5a) corresponds to the hydroxyl groups in their phenol moiety. The large width of the band was due to intra- and inter-molecular hydrogen bonding interactions typical of such groups [38]. The band was very similar in shape and strength for all pure gallates, since they contain identical number of hydroxyl groups. However, reaction with the enzyme decreased this band due to disappearance of such groups, and made it even broader, possibly by an increased number of hydrogen-bonding interactions in the remaining groups. This latter finding was evident for all compounds. Enzyme modification may have caused the formation of carboxyl groups (as explained in the previous section); hydroxyl functions in carboxyl groups typically establish strong hydrogen bonds (noticeable by the shift of the band to lower frequencies).

The carbonyl group in pure gallates (Figure 5c) absorbed at different frequencies (see table 1). In pure GA, the carbonyl peak in the acidic group appeared at 1700 cm^{-1}, as described elsewhere. Besides MG, the carbonyl peak in the gallates shifted to gradually-decreasing wavenumbers. Specific substituents may cause variations in absorption frequencies, producing the expansion of carbonyl (C=O) stretch region. Thus, the stretch of conjugated C=O (as in gallates), lie at lower wavenumbers. This conjugation combined with the possible hydrogen-bonding interactions may have reduced the stretch frequency of the C=O groups. Results suggest that longer alkyl chains tend to decrease the vibrational frequency of the C=O group, possibly by the increased probability of interaction of this group with a larger number of hydrogen atoms in the chains, leading to the weakening of the bond vibration.

The spectrum of SG was unchanged by the action of laccase: its peaks were identical in shape and strength before and after reaction. This suggests that SG was not or slightly modified by the enzyme. In fact, the reaction product exhibited no color, which suggested that modification was not taking place. This is in contrast to what is observed for the other gallates, in which the resulting o-quinones upon enzymatic reaction usually undergo side reactions, either between one another or with other substances in their immediate vicinity, to form a variety of colored compounds [51–53]. Possibly, the long hydrocarbon chain of the SG molecule may have prevented the phenol moiety from accessing the catalytic site of the enzyme, thereby inhibiting its oxidation due to steric hindrance. In fact, some authors have suggested that the rate of oxidation of gallates by the enzyme tyrosinase decreases with increasing alkyl chain length of the substrate [54]. In the present work, similar effects were observed with laccase.

Enzymatic modification of GA, MG and EG caused their carbonyl peak to disappear. Longer gallates (PG, BG, OG and LG), exhibited the peak at the same frequency (1714 cm^{-1}), except in SG, which has not been modified. Therefore, the enzyme may have induced similar chemical changes in PG, BG, OG and LG. All pure gallates exhibited three well-defined peaks at the same vibrational frequencies in the range 1615–1450 cm^{-1}, corresponding to C=C-C vibrations in the aromatic ring (Figure 5c). The central peak (1540–1520 cm^{-1}) disappeared upon reaction with the enzyme (Figure 5d) and thus reflected the changes undergone by the aromatic ring. The peak at 1200 cm^{-1} in pure gallates (Figure 5c) might correspond to vibration of phenolic C–OH bonds, since all compounds exhibited one. Enzymatic modification caused the peak to disappear from the spectra for some (GA, MG, EG) and may be masked by the broad band at higher frequencies (about 1225 cm^{-1}) for others (PG, BG, OG and LG) (Figure 5d).

Proposed mechanisms for the bonding of enzyme-modified LG to cellulose

As explained in the first part of the present work, interactions between enzyme-modified LG and cellulosic materials increase their hydrophobic properties. As revealed by FTIR observations, the enzyme-modified LG molecule possesses a hydrophilic head with acidic moieties, and a hydrophobic alkyl chain. The open-ring carboxyl groups (Figure 4) confer enzyme-modified LG a strong anionic character; this is the main aspect which facilitates the retention of such molecules onto cationic cellulosic materials, as observed in previous works.

Based on previous considerations on paper sizing [55], effective hydrophobization of cellulosic materials with LG requires i. the hydrophilic head of LG to be oriented towards the fibres, and the hydrophobic tail outwards; ii. LG to be evenly distributed throughout the material; and iii. optimal spacing between LG molecules on the fibres, allowing an efficient water-repellent character. Since the most likely scenario for enzyme-modified LG is one where all the molecules in Figure 4 coexist, we propose several mechanisms for the grafting of enzymatically treated LG to cellulosic materials.

Mechanism 1. Physicochemical interaction

As found in previous studies [37], a significant increase in hydrophobic behaviour was obtained with cationic substrates, produced with compounds such as polyamidoamine–epichlorohydrin, cationic starch, or methyl glycol chitosan before treatment with enzyme-modified LG. The strong anionic character of enzyme-modified LG conferred by its carboxyl groups would facilitate interaction with the cationic groups in cellulose, and hence its functionalization (see Figure 6a).

Mechanism 2. Esterification reactions

The oxidized LG obtained in step (7) of Figure 4 would react with cellulose and water by esterification as depicted in Figure 6b. In our previous study [34] we found that heat treatments increase hydrophobicity; this effect can be ascribed to the removal of water, which is therefore essential to promote the esterification reactions. Under drying conditions, an ester may form between carboxyl groups in LG and hydroxyl groups in cellulose surfaces. Oxidized LG could even form a highly reactive cyclic anhydride (Figure 6b) which would facilitate grafting without heat treatment via a reaction mechanism similar to that for alkenyl succinic anhydrides (ASAs) used as sizing agents for cellulosic materials [56,57]. Minimizing hydrolysis of the enzyme-modified LG molecule to promote the grafting reactions entails efficiently removing water in the medium by drying. Oxidized LG moieties may attach to cellulose surfaces via Fischer's esterification reaction between hydroxyl surface groups in cellulose and carboxyl groups in enzyme-modified LG. In this mechanism, the sodium tartrate buffer used in the enzymatic reaction, which derives from tartaric acid, would act as a strong acid catalyst for the reaction promoting protonation of carboxyl groups (a reversible reaction); the composition of the reaction mixture or equilibrium position would be thermodynamically governed. Obtaining the ester in good yields would require efficient removal of water by drying. In fact, the presence or addition of water here is of some concern as the water can shift the reaction equilibrium away from the ester formation and towards the carboxylic acid.

By effect of the esterification, the hydrophobic tail of LG would adopt an appropriate orientation —which, as noted earlier, is a prerequisite for the development of hydrophobic effect.

Mechanism 3. Ring-opening grafting reactions

The reactions induced via lactone ring-opening involve a monomer, an initiator and a catalytic system. Since an alcohol (or hydroxyl group) is generally used as initiator, cellulose in its native form acts as a multifunctional trigger for the modification of cellulose or cellulose derivatives [58]. The cyclic product obtained in step (5) of Figure 4 is a lactone that could be grafted to cellulose via a ring-opening mechanism similar to the one shown for ε-caprolactones and lactic acids elsewhere [59–61].

In the grafting of cellulose using the conventional ring-opening polymerization a metal catalyst (usually stannous 2-ethylhexanoate, SnOct2) is commonly used; this makes the resulting cellulose products useless for biomedical and food packaging applications, and promoted the use of alternative, non-toxic organocatalysts since 2001 [62–64]. For example, Hafrén and Córdova [65] reported on the surface-initiated grafting of ε-caprolactone from a cellulose substrate using tartaric acid as catalyst. Remarkably, in the current work the buffer solution used to obtain the enzyme-modified LG was prepared from tartaric acid. Thus, the lactone formed in step (5) of Figure 4 could be grafted via ring-opening onto the cellulosic material under heating conditions, following similar mechanisms to those reported by Hafrén and Córdova. Heating is required for efficient grafting of lactones onto cellulosic materials; as shown in previous works [36], heat treatments boosted grafting of enzyme-modified LG onto cellulose. Figure 6c depicts the proposed mechanism for the surface grafting via lactone ring-opening. The mechanism differs from that reported elsewhere for the ring opening polymerization of ε-caprolactone in that there's no actual polymerization after the initial grafting, since there are no active ends in the grafted molecule.

Conclusions

The treatment of cellulosic substrates with enzyme-modified LG and other gallates produced a significant improvement in the water contact angle and $Cobb_{60}$ values. In addition, the water absorption of the material was significantly reduced, evidencing a strong hydrophobic behaviour. The SEM images of paper specimens treated with FS revealed the presence of LG particles attached to the fibres. An exhaustive FTIR analysis revealed that enzyme-modified LG consists mainly of open-ring acidic products, which may undergo further oxidation via various pathways. Strong H-bonding interactions were observed in the resulting molecules. By contrast, the hydrocarbon chain of LG remained unaltered. FTIR-ATR analysis of enzyme-modified gallate-family-compounds also revealed that their alkyl chain was unaltered upon enzymatic modification, while major changes were observed in the peaks of their phenolic moieties. Stretching vibrations in the carbonyl and hydroxyl groups were considerably altered by effect of H-bonding interactions in all pure and modified gallates. A plausible oxidation mechanism was developed for LG, and several alternatives for the grafting of such molecules to cellulose-based materials were proposed.

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Supporting information

Time/concentration influence on hydrophobicity of FS-treated sheets (Table S1 and Figure S1); SEM analysis of FS control products (Figure S2); Particle size measurements using ImageJ software (Figure S3); Hydrogen-bond interactions of pure and enzyme-modified LG (Figure S4 and S5).

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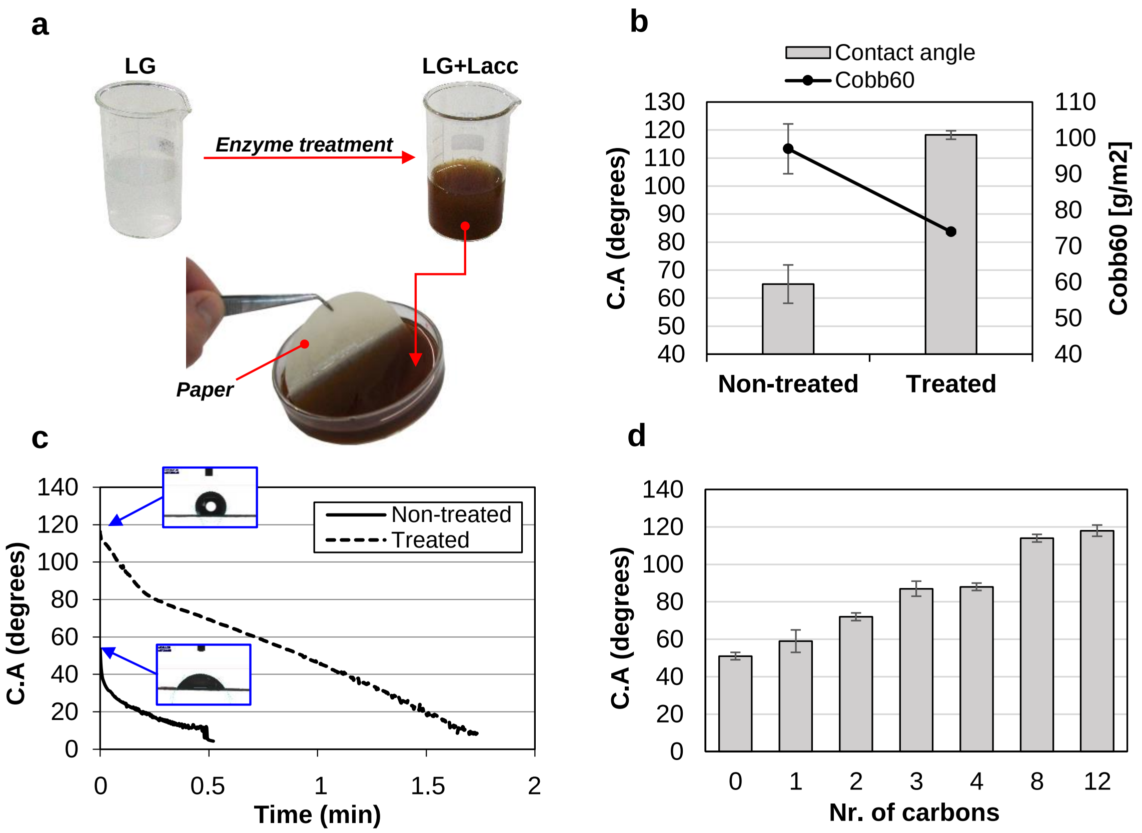
Table 1

Gallate	Carbonyl frequency
GA	1697 cm^{-1}
MG	1669 cm^{-1}
EG	1704 cm^{-1}
PG	1689 cm^{-1}
BG	1687 cm^{-1}
OG	1684 cm^{-1} (double)
LG	1666 cm^{-1}
SG	1668 cm^{-1}



a
LG
LG+Lacc
Enzyme treatment
Paper
b
Contact angle
Cobb60
C.A (degrees)
Cobb60 [g/m2]
130
120
110
100
90
80
70
60
50
40
Non-treated
Treated
c
140
120
100
80
60
40
20
0
C.A (degrees)
Non-treated
Treated
0
0.5
1
1.5
2
Time (min)
d
C.A (degrees)
0
1
2
3
4
8
12
Nr. of carbons

Figure 1

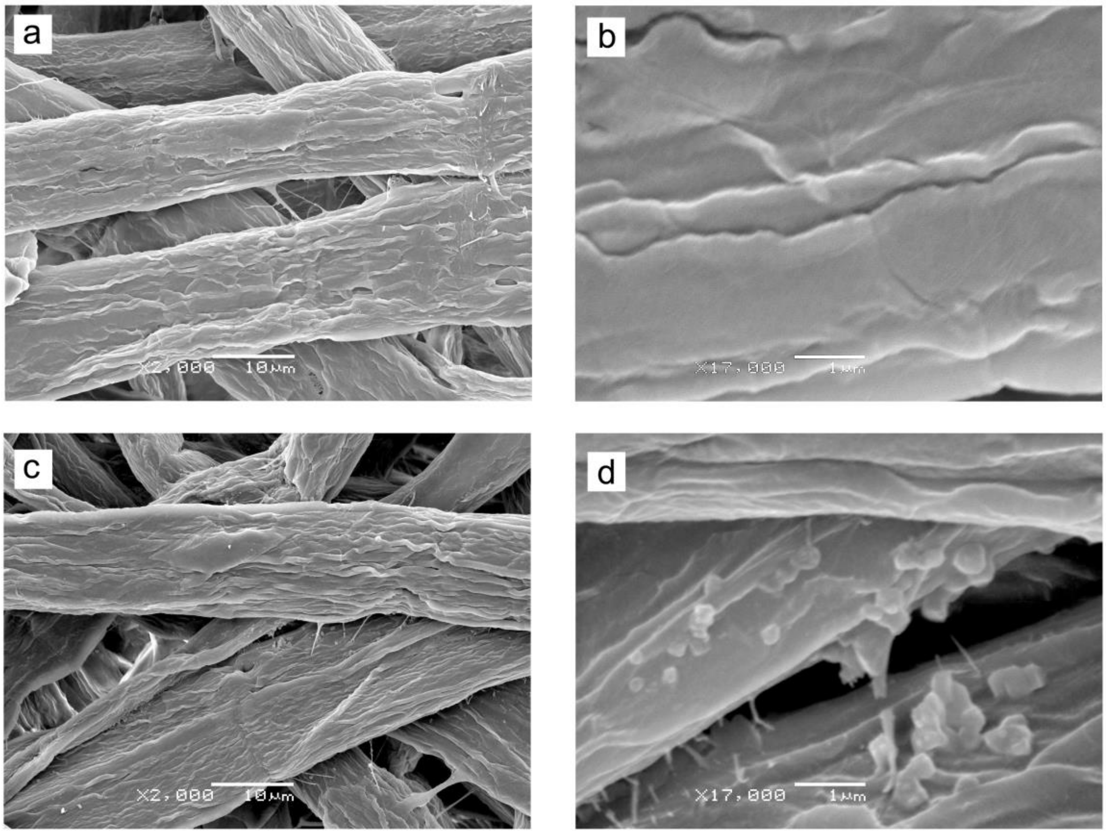


Figure 2

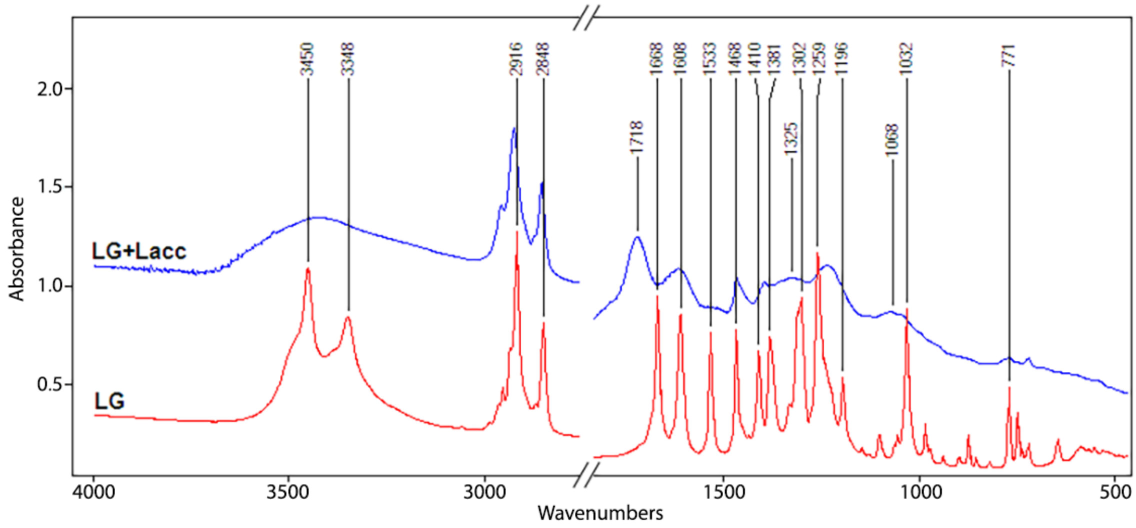

Absorbance
2.0
1.5
1.0
0.5
LG+Lacc
LG
4000
3500
3000
1500
1000
500
Wavenumbers
3450
3348
2916
2848
1718
1668
1608
1533
1468
1410
1381
1325
1302
1259
1196
1068
1032
771

Figure 3

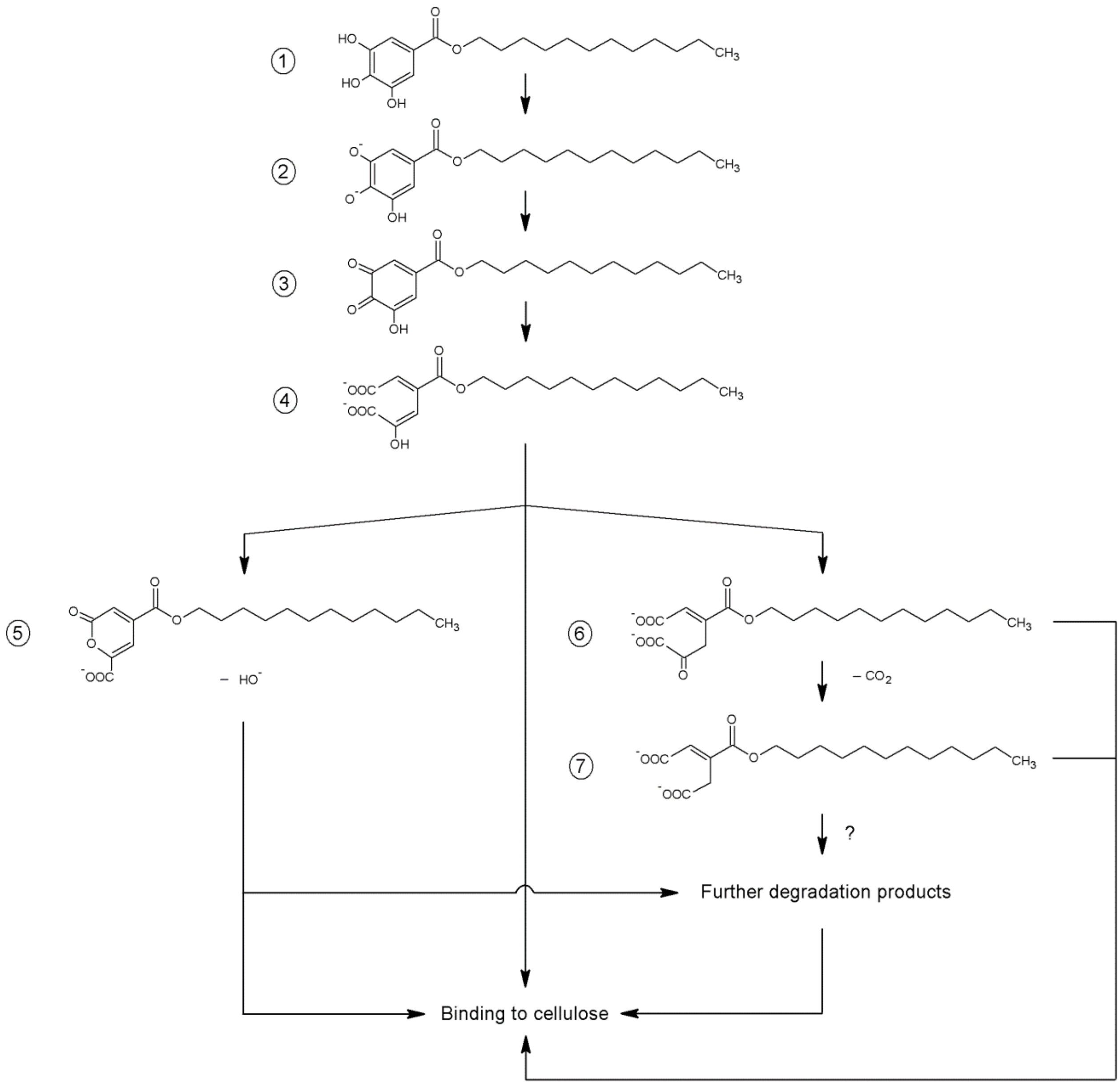

1
2
3
4
5
6
7
– HO⁻
– CO2
?
Further degradation products
Binding to cellulose

Figure 4

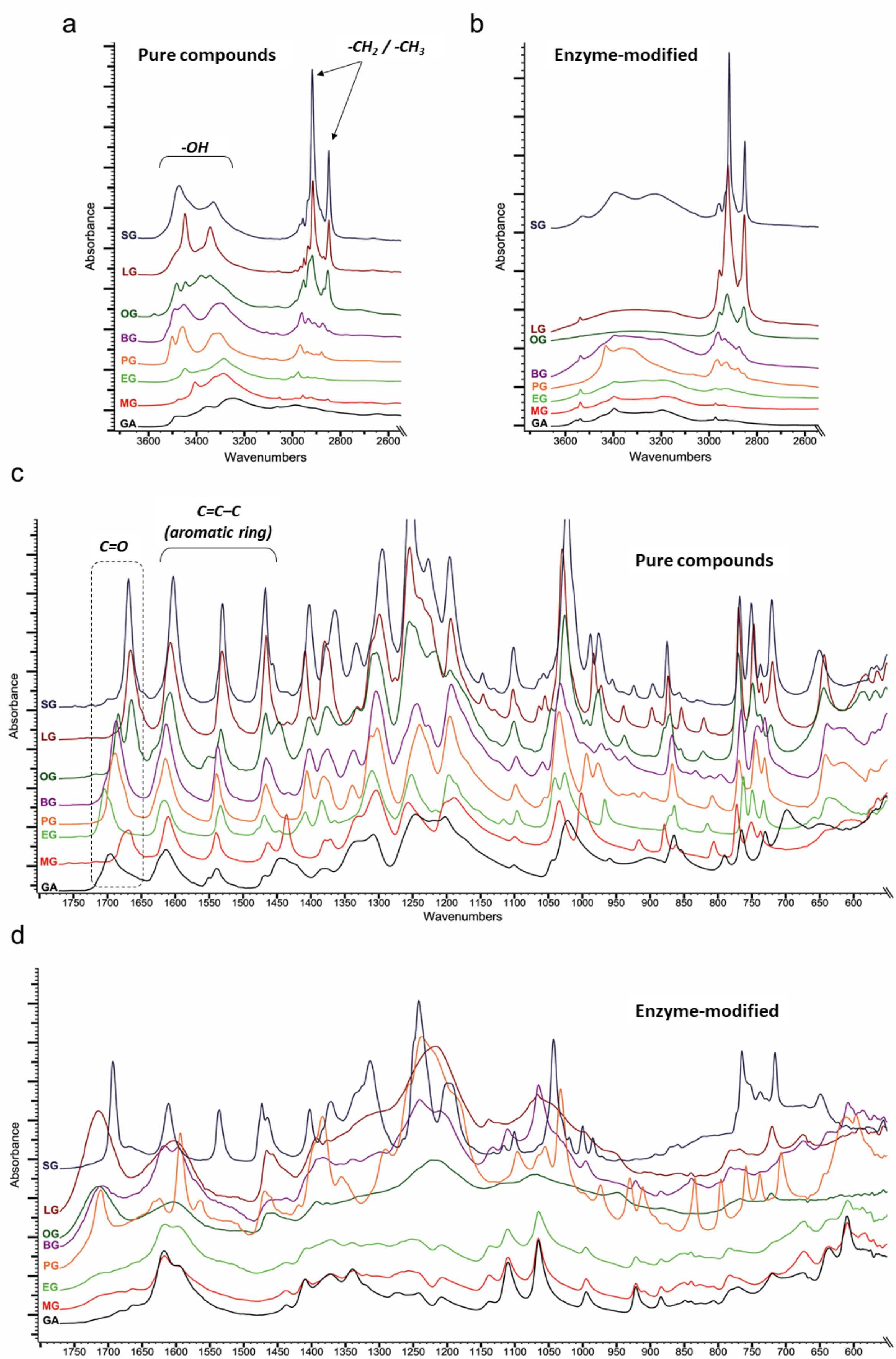

a
Pure compounds
-OH
$-CH_2$ / $-CH_3$
Absorbance
SG
LG
OG
BG
PG
EG
MG
GA
3600 3400 3200 3000 2800 2600
Wavenumbers
b
Enzyme-modified
Absorbance
SG
LG
OG
BG
PG
EG
MG
GA
3600 3400 3200 3000 2800 2600
Wavenumbers
c
C=O
C=C–C
(aromatic ring)
Pure compounds
Absorbance
SG
LG
OG
BG
PG
EG
MG
GA
1750 1700 1650 1600 1550 1500 1450 1400 1350 1300 1250 1200 1150 1100 1050 1000 950 900 850 800 750 700 650 600
Wavenumbers
d
Enzyme-modified
Absorbance
SG
LG
OG
BG
PG
EG
MG
GA
1750 1700 1650 1600 1550 1500 1450 1400 1350 1300 1250 1200 1150 1100 1050 1000 950 900 850 800 750 700 650 600
Wavenumbers

Figure 5

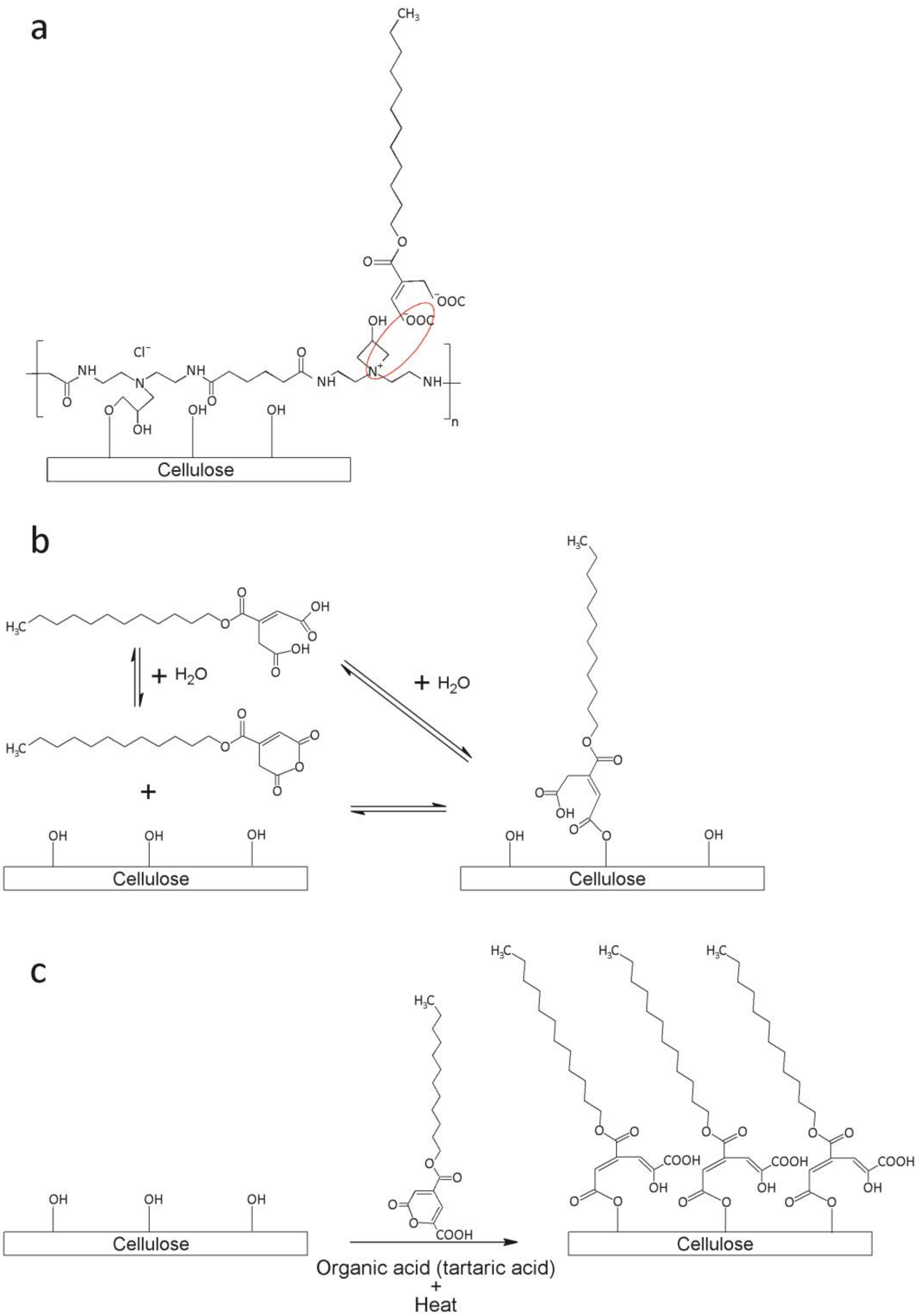

a
Cellulose
b
+ H2O
+ H2O
+
Cellulose
Cellulose
c
Cellulose
Organic acid (tartaric acid)
+
Heat
Cellulose

Figure 6

List of figure and table captions

Figure 1. Aqueous suspensions of LG before and after reaction with laccase, and treatment of paper sheets (a); water contact angle and $Cobb_{60}$ values of a 67 g/m2 paper material before and after treatment with FS (b); time-dependent evolution of the water CA of FS-treated and non-treated 67 g/m2 papers (c); and hydrophobic behavior evolution of paper sheets trated with enzyme-modified ingreasing alkyl-chain-length gallates (d).

Figure 2. SEM images of untreated eucalyptus sheets (a, b), and FS-treated sheets (c, d). The images on the left are macroscopic views of the paper specimens (2000x) and those on the right are close views of the surface of a single fibre (17,000x).

Figure 3. FTIR spectra for the LG before and after modification by the enzyme.

Figure 4. Proposed oxidation mechanism for LG.

Figure 5. FTIR spectra for pure (a) and enzyme-modified gallates (b) in the range 3600–2600 cm-1; FTIR spectrum of the pure (a), and enzyme-modified (b) gallates in the wavenumbers ranging from the 1750 to the 600 cm-1.

Figure 6. Physico-chemical interaction between PAAE-treated cellulose and an enzyme-modified LG moiety (a); attachment of enzyme-modified LG moieties to the surface of cellulose via Fischer’s esterification reaction and drying (b); and tartaric acid-catalyzed ROP of the enzyme-modified LG lactone from cellulose fibers (c).

Table 1. FTIR absorption frequencies for the carbonyl group (C=O) in the pure gallates.