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Template-mediated synthesis and bio-functionalization of flexible lignin-based nanotubes and nanowires

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Abstract

Limitations of cylindrical carbon nanotubes based on the buckminsterfullerene structure as delivery vehicles for therapeutic agents include their chemical inertness, sharp edges and toxicological concerns. As an alternative, we have developed lignin-based nanotubes synthesized in a sacrificial template of commercially available alumina membranes. Lignin is a complex phenolic plant cell wall polymer that is generated as a waste product from paper mills and biorefineries that process lignocellulosic biomass into fuels and chemicals. We covalently linked isolated lignin to the inner walls of activated alumina membranes and then added layers of dehydrogenation polymer onto this base layer via a peroxidase-catalyzed reaction. By using phenolic monomers displaying different reactivities, we were able to change the thickness of the polymer layer deposited within the pores, resulting in the synthesis of nanotubes with a wall thickness of approximately 15 nm or nanowires with a nominal diameter of 200 nm. These novel nanotubes are flexible and can be bio-functionalized easily and specifically, as shown by in vitro assays with biotin and Concanavalin A. Together with their intrinsic optical properties, which can also be varied as a function of their chemical composition, these lignin-based nanotubes are expected to enable a variety of new applications including as delivery systems that can be easily localized and imaged after uptake by living cells.

Online supplementary data available from stacks.iop.org/Nano/23/105605/mmedia

1. Introduction

Carbon nanotubes based on the buckminsterfullerene structure but with a cylindrical shape can, in principle, be used as universal delivery vehicles for therapeutic agents [1–5], but their chemical inertness, sharp edges and toxicological concerns limit their versatility and suitability [6–13]. As alternatives, nanotubes can also be formed from polymeric
materials such as polyelectrolytes [14], collagen [15] and poly(3,4-ethylenedioxythiophene) [16] via template-mediated synthesis using nanoporous membranes containing arrays of aligned cylindrical pores [17, 18]. This approach can result in either polymer-functionalized nanoporous membranes or polymer-based nanotubes released from a sacrificial template [19]. The development of polymer nanotubes is promising for biomedical and biotechnological applications because of their greater biocompatibility [20, 21]. However, commercial applications of synthesized polymer-based nanotubes are currently limited by the fact that these structures do not maintain their shape indefinitely [22, 23].

In an attempt to identify additional materials suitable for the production of biocompatible nanotubes whose physico-chemical properties can be controlled and tailored, we investigated the potential of lignin. Lignin is a complex phenolic plant cell wall polymer formed by the oxidative coupling of hydroxycinnamyl alcohols and related compounds, including hydroxycinnamaldehydes and hydroxycinnamic acids [24, 25] (supplemental figure 1 available at stacks.iop.org/Nano/23/105605/mmedia). The composition of lignin varies naturally among species, tissues within a given plant, and as a function of plant development, in order to provide the most suitable biological and physical properties, which include providing structural support, a hydrophobic surface that facilitates water transport through vascular tissues and a mechanical barrier against invading microbes and insects. A synthetic lignin polymer referred to as a dehydrogenation polymer (DHP) can be synthesized in vitro by combining hydroxycinnamyl alcohols and oxidative enzymes [26–29]. Lignin is an abundantly available, low-cost waste product from biorefineries that hydrolyze cell wall polysaccharides from renewable resources, such as woody biomass, corn stover and wheat straw, and convert the monosaccharides into fuels and chemicals [30]. The number of biorefineries is expected to increase as a result of the desire to reduce both the dependence on imported fossil fuels and the net emission of greenhouse gases. Since the presence of lignin in the biomass lowers the biodegradability and biocompatibility of the resulting nanotubes, it is feasible to tailor the physico-chemical and biological properties of lignin-based materials through genetic approaches, which offers the potential of custom templates for nanomaterials. By combining the principle of combinatorial chemistry of lignin polymer synthesis with template synthesis of nanostructures we were able to synthesize nanotubes and nanowires whose structural and chemical features can be easily tailored by varying the monomer supply and synthesis conditions. By controlling the rate of synthesis, we were able to effectively control the thickness of the polymeric layer deposited within the pores, and therefore the strength of the nanotube network structure, resulting in the synthesis of either nanotubes with an approximate wall thickness of 15 nm or nanowires with a 200 nm nominal diameter. By varying the composition of the monomers we were able to modify the optical properties of the resulting nanotubes. We also demonstrate how, as a result of the many functional and reactive groups in the lignin, lignin-based nanotubes are highly amenable to bio-functionalization with a variety of biologically active molecules, which enables specific recognition of biological targets and expands the opportunities for the targeted delivery of therapeutic agents in humans and animals.

2. Experimental details

2.1. Synthesis of lignin nanotubes

Commercially available nanopore alumina membranes (60 µm thick, nominal pore diameter 200 nm) were obtained from Whatman and boiled for 15 min at 100 °C in 30% (v/v) hydrogen peroxide to increase the number of reactive hydroxyl groups on the surface, then rinsed by boiling in deionized water for 15 min. The membranes were dried under nitrogen and functionalized with amino groups by immersing the alumina membranes in 2% (v/v) (3-aminopropyl)-triethoxysilane in ethanol for 1 h. The membranes were copiously rinsed with 100% ethanol, air-dried and oven-cured overnight at 100 °C. Thioglycolate lignin was extracted from approximately 20 mg maize brown midrib1 (bmi1) stover, which contains lignin with increased levels of coniferaldehyde [36]. The stover had been pre-extracted in 50% (v/v) ethanol at 60 °C and oven-dried (50 °C). The lignin pellet was rinsed several times with deionized water and dissolved in 0.5 M NaOH [37]. A 2 ml volume of solubilized thioglycolate lignin was adjusted to pH 8.0 with 2 M HCl and diluted with 50 mM sodium phosphate buffer pH 7.4 containing 1 M NaCl to a final volume of 10 ml. Amino-silane-derivatized membrane segments of approximately 1.5 cm² were immersed in 2 ml of the phosphate buffer containing thioglycolate lignin and incubated for 24 h at 4 °C. The membranes were copiously rinsed with 100% ethanol and dried under nitrogen. The resulting coating served as a template onto which a dehydrogenation polymer (DHP) was deposited via oxidative coupling of hydroxycinnamyl alcohols (coniferyl alcohol), hydroxycinnamaldehydes (coniferaldehyde) or hydroxycinnamic acids (ferulic acid, p-coumaric acid), either neat or in mixtures. Each sample of lignin-functionalized alumina template was immersed in a 3 ml solution containing 25 mM of the particular monomer (mixture) dissolved in 100 mM potassium phosphate buffer, pH 6.0. Then 0.2 ml of freshly prepared solution of 0.2 mg ml⁻¹ horseradish peroxidase (188 purpurogallin units/mg) in 0.1 M potassium phosphate buffer, pH 6.0, was added, followed by drop-wise addition at room temperature of 20 µl 200 mM hydrogen peroxide every 4 min for a period of 2 h. The reaction solution was removed and
Figure 1. Schematic illustration of the synthesis of lignin nanotubes. (a) Activation of alumina membrane with APTES; four vertically oriented pores are shown in this image, but for the sake of clarity the reactive sites are only shown on one of the pore walls. The inactivated (sputter-coated) upper and lower surfaces of the membrane are indicated by the dark-gray caps. (b) Schiff’s base reaction between the amino groups on the APTES-activated membrane and aldehyde moieties in the thioglycolate lignin forms a base layer; only one pore wall is displayed. (c) Hydroxycinnamic acids, hydroxycinnamaldehydes or hydroxycinnamyl alcohols are added and (d) polymerize onto the lignin base layer through a reaction catalyzed by horseradish peroxidase (HRP)/H$_2$O$_2$. DHP = dehydrogenation polymer.

the alumina template was copiously washed with 100 mM potassium phosphate buffer pH 6.0. Additional washes with 100% ethanol were carried out and the membranes were dried under nitrogen. A total of four layers of DHP were deposited onto the lignin base layer. In order to release the lignin-based nanostructured material, the alumina template was dissolved by immersion in 5% (v/v) phosphoric acid containing 0.5% (v/v) Triton X-100 surfactant. After 4 h the template was completely dissolved and the released nanotubes were sonicated for 15 min. The nanotubes were collected by centrifugation (4000 rpm, 4°C) and washed four times with water containing 0.05% (v/v) Triton X-100.

2.2. Physico-chemical characterizations

Scanning electron microscopy (SEM) and energy dispersive x-ray spectroscopy (EDS) were performed using a JEOL JSM 6335F field-emission scanning electron microscope linked to an Si(Li) energy dispersive spectrometer (ISIS system from Oxford Instruments, Ltd). For imaging, 50 µl of a solution containing the lignin nanotubes was pipetted onto nitrocellulose, dried at room temperature and mounted on alumina stubs. The samples were Au/Pd sputter-coated for imaging purposes and coated with carbon using a carbon evaporator for the EDS analysis. Glass slides were carbon-coated and mounted on alumina stubs for SEM imaging. Transmission electron microscopy (TEM) was performed on lignin nanotubes pipetted onto TEM Cu grids with a graphite support film. A JEOL 2010F field-emission TEM was used to acquire the images. Pyrolysis-GC-MS was performed on 1 mg dried nanotubes placed in a quartz cup and introduced into a 1079 programmable temperature vaporization (PTV) injector (Varian, Walnut Creek, CA) mounted on a Varian 3800 gas chromatograph coupled to a Varian 1200 mass spectrometer. The nanotubes were pyrolyzed at 450°C under helium and the resulting thermal degradation products were separated on a capillary column (25 m, 0.32 mm i.d. fused silica coated with SGE-BPX5), which was inserted in the PTV outlet set at 325°C. Gas chromatography was carried out with helium as the carrier gas at a constant flow rate of 2.5 ml min$^{-1}$. The split ratio was 1/20. The GC program started at 70°C for 1 min, followed by a temperature increase to 220°C at a rate of 4°C min$^{-1}$ followed by a temperature increase to 325°C at 6°C min$^{-1}$. The mass spectrometer was operated at 1.2 kV. The mass range included mass-to-charge ratio (m/z) 45–350 and was scanned every 0.20 s. Data were acquired using Varian WS software. Compound identification was based on a combination of the NIST 08 mass spectral library, and Ralph and Hatfield [25].

2.3. Quantification of lignin nanotubes used for bio-functionalization experiments

Nanotubes produced as described above from equimolar amounts of ferulic and p-coumaric acid were liberated via acid dissolution of the alumina membrane template, collected via centrifugation, washed in ddH$_2$O and suspended in 2 ml ddH$_2$O. Nanotubes were quantified as lignosulfonic acid equivalents, based on a calibration with lignosulfonic acid (Sigma Aldrich) with an average molecular weight of 12 000 g mol$^{-1}$, dissolved in 1 M NaOH. Absorbance was measured spectrophotometrically at 280 nm on a SpectraMax M5 plate reader.
2.4. Amino-derivatization of glass platforms

Pieces of glass microscope slides with an approximate area of 1 cm\(^2\) were thoroughly cleaned with freshly prepared piranha solution (3:1 concentrated sulfuric acid and 30% (v/v) hydrogen peroxide, 65 °C for 45 min). The cleaned glass slides were placed in a Teflon beaker containing 50 ml ethanol and 1 ml APTES, pH adjusted to 4.5 with acetic acid, and incubated for 30 min to functionalize the glass with amino groups. The slides were extensively rinsed with ethanol to remove excess silane, dried under a nitrogen stream, baked overnight at 105 °C and stored under a nitrogen atmosphere until use, for a maximum of five days.

2.5. Direct immobilization of lignin nanotubes onto amino-derivatized glass platforms

The amino-derivatized glass surfaces were extensively rinsed with 100 mM MES buffer pH 7.0 containing 500 mM NaCl. The upper surface of each glass section was covered with 0.2 ml MES/NaCl buffer containing 5 mg ml\(^{-1}\) of the activating agent N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC; Sigma Aldrich), which activates carboxyl groups, thus catalyzing the formation of amide bonds (supplemental figure 3 available at stacks.iop.org/Nano/23/105605/mmedia), and 0.25 ml of a suspension of lignin nanotubes (in water containing 0.05% (v/v) Triton X-100) with a concentration of 90 \(\mu\) M lignosulfonic acid equivalents. Following incubation at 4 °C for 4 h, surfaces were rinsed copiously with 0.1 M phosphate-buffered saline (PBS) pH 7.4 and ddH\(_2\)O, and viewed under an Olympus MVX10 fluorescence microscope using the red fluorescence filter to detect the intrinsic signal from the lignin nanotubes. Negative controls were identical as described above, except that EDC was omitted, thus limiting the formation of amide bonds between the amino-functionalized glass and the carboxyl groups on the exterior of the nanotubes.

2.6. Immobilization of lignin nanotubes functionalized with avidin onto glass functionalized with desthiobiotin

Derivatization of the glass surface with desthiobiotin was identical as described above, except that 1 mg ml\(^{-1}\) desthiobiotin (Sigma Aldrich) was added to the 100 mM MES/500 mM NaCl buffer containing 5 mg ml\(^{-1}\) EDC. After 4 h incubation at 4 °C the desthiobiotin-derivatized surfaces were rinsed with PBS pH 7.4. Lignin nanotubes were derivatized concurrently with Avidin-fluorescein isothiocyanate (FITC) (Sigma Aldrich) by combining 2 ml MES/NaCl buffer containing 5 mg ml\(^{-1}\) EDC, 2 mg Avidin-FITC and 0.5 ml of a suspension of lignin nanotubes (90 \(\mu\) M lignosulfonic acid equivalents). A 0.2 ml volume of the avidin-FITC-functionalized nanotube suspension was placed on the surface of the desthiobiotin-derivatized glass and incubated for 4 h at 4 °C to allow binding. Afterwards, the surfaces were rinsed copiously with PBS pH 7.4 and ddH\(_2\)O, and visualized under a fluorescence microscope using an RFP or GFP filter to detect the nanotubes or the FITC, respectively.
Figure 3. EDS microanalysis for the local chemical composition of the lignin nanotube. (a) Partial dissolution of the alumina template. (b) Free lignin nanotube after complete dissolution of the template. (c) Pyrogram of lignin-based nanotubes synthesized using a 1:1 molar ratio of 

\[ p \text{-coumaric acid and ferulic acid. Peaks representing compounds for which the identity could be unambiguously established based on the NIST08 spectral library, and Ralph and Hatfield [25] are as follows, with the } m/z \text{ values of the main ions, including the molecular ion } [M]+, \text{ and the most likely origin of the compound in parentheses: (1) 4-ethylphenol (} m/z 122 [M]+, 107; p\text{-coumaric acid), (2) 2-methoxy-4-methylphenol (} m/z 138 [M]+, 123; ferulic acid, residues derived from coniferyl alcohol in lignin base layer), (3) 4-vinylphenol (} m/z 120 [M]+, 91; p\text{-coumaric acid), (4) 4-ethyl-2-methoxyphenol (} m/z 152 [M]+, 137; ferulic acid, residues derived from coniferyl alcohol in lignin base layer), (5) 4-vinylguaiacol (} m/z 150 [M]+, 135; ferulic acid), (6) vanillin (} m/z 152 [M]+, 151; coniferaldehyde in lignin base layer), (7) 2-methoxy-4-propylphenol (} m/z 166 [M]+, 137; ferulic acid; residues derived from coniferyl alcohol in lignin base layer), (8) ferulic acid methyl ester (} m/z 208 [M]+, 177, 145; ferulic acid).}

Negative controls were identical as described above, except that EDC was omitted in the functionalization reaction of the nanotubes.

2.7. Immobilization of lignin nanotubes functionalized with anti-concanavalin A onto glass functionalized with concanavalin A

Amino-derivatized glass surfaces were extensively rinsed with 0.1 M PBS pH 7.4, incubated for 2 h at room temperature in a beaker containing 20 ml 4% glutaraldehyde in 0.1 M PBS pH 7.4, washed in PBS and ddH₂O, and dried and stored under a nitrogen atmosphere until use. The upper surface of each piece of amino-linked-derivatized glass was incubated at 4 °C for 4 h with 0.2 ml PBS containing 1 mg ml⁻¹ concanavalin A (Sigma Aldrich). Concurrently, lignin nanotubes were functionalized in 2 ml MES/NaCl buffer containing 5 mg ml⁻¹ EDC, 1 mg ml⁻¹ anti-concanavalin A (Sigma Aldrich) and 0.5 ml of a suspension of lignin nanotubes (90 μM lignosulfonic acid equivalents). After rinsing the concanavalin A-derivatized glass surfaces with PBS, 0.2 ml of the anti-concanavalin A-functionalized nanotube suspension was placed on the upper surface of each piece of glass and incubated at 4 °C
for 4 h. Afterwards, the surfaces were rinsed copiously with PBS and ddH$_2$O, and visualized under a fluorescence microscope using the RFP filter. Negative controls were identical as described above, except that EDC was omitted in the functionalization reaction of the nanotubes.

3. Results and discussion

**Synthesis of lignin nanotubes**

A schematic illustration of the reaction mechanism for the template synthesis of lignin nanotubes is shown in figure 1. The pore walls of commercially available alumina membranes ($10^9$ pores cm$^{-2}$) were first activated with (3-aminopropyl)-triethoxysilane (APTES). Aldehyde-rich lignin was isolated from the stover of brown midrib1 (bml) mutant maize (Zea mays L.) [36] using the thioglycolic acid procedure [37] and subsequently attached to the walls of amino-silane-functionalized pores of an alumina membrane via a Schiff’s base reaction. The covalently attached lignin base layer inside the membrane pores served as a template onto which a layer of dehydrogenation polymer (DHP; ‘synthetic lignin’) was deposited via a horseradish-peroxidase-catalyzed reaction using either hydroxycinnamyl alcohols (monolignols), hydroxycinnamylaldehydes or hydroxycinnamic acids [38, 39].

Figure 2 shows a scanning electron micrograph of the initial synthesis of lignin nanotubes based on oxidative coupling of ferulic acid (4-hydroxy, 3-methoxycinnamic acid) and $p$-coumaric acid (4-hydroxycinnamic acid; structures provided in supplemental figure 1 available at stacks.iop.org/Nano/23/105605/mmedia) in a 1:1 molar ratio onto the base layer of thioglycolate lignin. In addition to the deposition of lignin inside the pores of the membrane, material was deposited on the top and bottom surfaces of the membrane. This resulted in the formation of bundles of nanotubes or nanowires that could not be separated into individual structures unless the top and bottom layers were removed via mechanical abrasion with a razor blade and cotton.
swabs impregnated with dichloromethane. Even after removal of the top and bottom layers, the nanotubes continued to form bundles, some of which were intertwined, most likely due to the presence of strong van der Waals interactions between adjacent nanotubes. The protocol was improved by sputter-coating the top and bottom surfaces of the membrane with Au/Pd prior to functionalization, rendering them inert to the reaction with APTES. In addition, surface tension during the dissolution of the membrane was minimized with the addition of the surfactant Triton X-100. A sonication step was included to maximize the disruption of intermolecular attractive forces involved in the nanotube aggregation. We suspect that the benzene rings and alkyl chains of the Triton X-100 are able to effectively adsorb onto the tubes through the formation of aromatic stacking interactions during the process of nanotube release from the membrane, thus stabilizing the individual nanotubes [40, 41].

Structural and chemical characterization of lignin nanotubes and nanowires

Gross chemical (elemental) composition of the lignin-based nanotubes was determined with EDS microanalysis and revealed that the examined nanostructure is essentially composed of carbon, oxygen and sulfur (figures 3(a) and (b); supplemental figure 2 available at stacks.iop.org/Nano/23/105605/mmedia). The presence of these elements is consistent with the chemical composition of the thioglycolate lignin (C, H, O, S). Quantification of the individual elements cannot be performed because the specimens are carbon-coated as part of the analysis, and furthermore susceptible to surface contamination by water and carbonate groups (from CO₂ in the air). In addition, there are limitations associated with the measurement of very light elements using EDS [42]. The chemical composition of the nanotubes determined with pyrolysis-GC-MS confirmed that p-coumaric and ferulic acid were the major components as shown by the large peaks representing their respective breakdown products, 4-vinylphenol (m/z 120 [M⁺], 91) and 4-vinylguaiacol (m/z 150 [M⁺], 135), [25, 45] (figure 3(c)). The presence of the lignin base layer derived from the stover of the maize bm1 mutant is evident from the presence of vanillin (m/z 152 [M⁺], 151), a breakdown product of coniferaldehyde [45].

Next, the effects of monomer composition and monomer ratio on the physico-chemical properties of the lignin-based carbon nanotubes were investigated. Nanotubes derived from coniferyl alcohol, coniferaldehyde, ferulic acid, p-coumaric acid (structures provided in supplemental figure 1 available at stacks.iop.org/Nano/23/105605/mmedia) and mixtures of ferulic acid and p-coumaric acid (in 5:1, 5:3, 3:5 and 1:5 molar ratios) were synthesized with the improved protocol. Striking differences in the color of the deposits on the membranes were apparent (figure 4), which are consistent with reports on in vitro synthesized DHP [27]. Furthermore, the nanotubes formed inside the membranes fluoresced when excited with UV radiation, consistent with the autofluorescence properties of natural lignin [43]. This attribute will be of great practical value to monitor the transport and/or location of lignin nanotubes in biological systems. The chemical composition of the nanotubes determined with pyrolysis-GC-MS reflected the variation in monomer composition (figure 5).

In order to assess the impact of monomer composition of the overall structure of the nanotubes TEM and SEM were employed to measure wall thickness and nanotube surface
Figure 6. SEM ((a)–(d) and (f)–(i)) and TEM ((e), (j)) images of lignin nanostructures synthesized with 5:1 (a)–(e) and 1:5 (f)–(j) molar ratios of ferulic acid and $p$-coumaric acid, respectively. Images (a)–(e) represent sequential phases of membrane dissolution leading to the formation of free nanotubes, whereas images (f)–(j) show the corresponding stages leading to the formation of free nanowires. Scale bars are as follows: (a) 20 $\mu$m, (b) 7.5 $\mu$m, (c) 4.29 $\mu$m, (d) 1 $\mu$m, (e) 0.2 $\mu$m, (f) 15 $\mu$m, (g) 12 $\mu$m, (h) 2.73 $\mu$m, (i) 2.73 $\mu$m and (j) 0.2 $\mu$m.
Figure 7. UV-fluorescence images (labeled ‘test’) of bio-functionalized template-synthesized lignin nanotubes (TSLNTs) immobilized and visualized on an amino-derivatized glass platform. (a) Direct immobilization of lignin nanotubes onto glass; 1000 ms exposure, RFP filter (570–625 nm) to detect signal from nanotubes. (b) Immobilization of lignin nanotubes functionalized with avidin-FITC onto glass functionalized with desthiobiotin; 1000 ms exposure, GFP filter (495–540 nm) to detect signal from FITC and (c) immobilization of lignin nanotubes functionalized with anti-concanavalin A onto glass functionalized with concanavalin A; 1000 ms exposure, RFP filter. The images labeled ‘blank’ are UV-fluorescence images obtained with lignin nanotubes that were not functionalized. Schematic diagrams representing the bio-molecular recognition are displayed above and below the corresponding microscope images. (d) SEM image of a representative section of the NH$_2$-derivatized glass slide after incubation with activated nanotubes (EDC chemistry) and washing in PBS buffer; scale bar equals 50 µm; (e) SEM image of a representative section of the NH$_2$-derivatized glass slide after incubation with underivatized nanotubes and washing in PBS buffer; scale bar equals 50 µm; (f) SEM image of a representative section of the concanavalin A-derivatized glass slide after incubation with nanotubes bio-functionalized with anti-concanavalin A antibodies, and washing in PBS buffer; scale bar equals 50 µm. (g) SEM image of a representative section of the concanavalin A-derivatized glass slide after incubation with underivatized nanotubes and washing in PBS buffer; scale bar equals 100 µm. Note that the negative controls show a larger surface area of the glass to underscore the absence of nanotubes.
structure. Given that the pore size of the template is known, and that TEM is performed in vacuo, hollow nanotubes will flatten out and appear wider than the pore size, whereas solid nanowires will have diameters equal to the pore size of the template. Figure 6 shows SEM and TEM images of nanostructures prepared with 5:1 (figures 6(a)–(e)) and 1:5 (figures 6(e)–(j)) molar ratios of ferulic acid: \( p \)-coumaric acid, respectively. These studies revealed that wall thickness was greater with the higher proportion of \( p \)-coumaric acid in the reaction. The synthesis carried out with a higher proportion of ferulic acid resulted in hollow nanotubes. This observation demonstrates that the structural characteristics of the nanotubes are under simple chemical control. We hypothesize that this feature reflects a strong morphological dependence on the rate of the polymerization reaction, with \( p \)-coumaric acid being more reactive because of its ability to form crosslinks at both the C3 and C5 positions of the phenolic ring, whereas ferulic acid does not have the C3 position available for crosslinks. In addition, the formation of an intermolecular hydrogen bond between the methoxy oxygen and the phenolic hydrogen may further reduce the reactivity of ferulic acid during the enzyme-mediated formation of the corresponding radical. The relationship between the chemical composition of the lignin and physical attributes of the resulting structure is consistent with what has been observed in plants: genetic perturbation of lignin subunit composition results in variation in the structure of water-conducting xylem vessels [44, 45].

**Bio-functionalization of lignin nanotubes**

In order for lignin nanotubes to be compatible with biological systems, for example as carriers of bioactive compounds to specific target cells or organs within the human body, it will be critical to be able to bio-functionalize the nanotube surfaces. The rich diversity in chemical bonds and functional groups present in the lignin structure enables multiple approaches for surface modification. Alternatively, the lignin nanotubes can be designed with surface properties tailored to a specific function. As a proof of concept, we bio-functionalized-lignin nanotubes synthesized from a 1:1 molar ratio of \( p \)-coumaric acid and ferulic acid (as described above) and demonstrated specific immobilization onto an amino-functionalized glass platform. Three different systems were examined: direct immobilization of nanotubes on the glass surface, molecular recognition of desthiobiotin with avidin and of concanavalin A with anti-concanavalin A antibodies (figure 7). The lignin nanotubes were functionalized using EDC as an activating agent of the carboxyl groups present in the lignin, so that
amide bonds could be formed with the amino groups on either the glass (direct immobilization) or the proteins (avidin; anti-concanavalin A). By omitting EDC from the functionalization reaction, any observed interactions of the nanotubes with the glass surface would be the result of non-specific binding. The intrinsic fluorescence of the lignin nanotubes permitted a label-free detection under the fluorescence microscope (figures 7(a) and (c)). However, FITC-labeled proteins were also employed to obtain additional evidence in support of the bio-molecular recognition reactions (figure 7(b)). The negative control showed a background fluorescence resulting from the binding of FITC-labeled avidin to desthiobiotinylated glass, whereas the desthiobiotinylated glass surface onto which the bio-functionalized nanotubes had been deposited showed distinct foci representing the binding of nanotubes. The observations based on fluorescence were corroborated using SEM (figures 7(d)–(f)), which showed individual nanotubes on the glass surface only when the nanotube functionalization reaction included EDC. The lack of detectable red fluorescence in the negative controls in which the cross-linking agent EDC was omitted, and the lack of nanotubes on the glass surface when viewed under SEM (only salt deposits are visible) clearly demonstrate that lignin nanotubes can be easily functionalized and are able to recognize and bind to targets in a specific manner. Given that nanowires have exterior surfaces with similar structure, due to the presence of the lignin base layer, bio-functionalization of nanowires is expected to result in similar results.

4. Conclusions

Template synthesis is a versatile technique to produce nanotubes, nanowires and other nanostructured materials. This technology was pioneered by Martin and colleagues [17, 19] to synthesize nanotubes of varying size by using different polymeric substrates. Here, we have demonstrated the ability to synthesize lignin nanotubes with an alumina template using a naturally biodegradable and biocompatible plant cell wall polymer. We have further shown that the nature of the monomers used to form the wall of the tubes has a strong effect on both the optical and physical parameters of the nanotubes. This means that optical properties and wall thickness can be fine-tuned for specific applications. Further parameters that may affect these properties, and the topic of follow-up studies, are the source of the lignin (gymnosperm and angiosperm woody species, herbaceous angiosperm dicots and monocot grasses), the lignin isolation procedure (mineral acids, alkaline solutions, ionic liquids), the amount of lignin used to form the base layer, and the pore size and thickness of the membrane. The ability to bio-functionalize these structures so that they can recognize and specifically bind to target molecules, combined with their pliable nature, has the potential to make these nanotubes uniquely suited for biomedical applications, such as the delivery of therapeutic agents to specific cells or organs. Conversely, functionalized nanowires may be of value to impede or modify physical and/or chemical interactions between ligands and receptors in living systems. The use of lignin nanomaterials derived from the waste stream of biorefineries as high-value co-products has the potential to offset biomass processing costs, thereby making the production of renewable fuels more cost-effective.

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