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Activation of Crystalline Cellulose Surfaces through the Chemoenzymatic Modification of Xyloglucan

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Abstract: Cellulose constitutes an important raw material for many industries. However, the superb load-bearing properties of cellulose are accompanied by poor chemical reactivity. The hydroxyl groups on cellulose surfaces can be reacted but usually not without loss of fiber integrity and strength. Here, we describe a novel chemoenzymatic approach for the efficient incorporation of chemical functionality onto cellulose surfaces. The modification is brought about by using a transglycosylating enzyme, xyloglucan endotransglycosylase, to join chemically modified xyloglucan oligosaccharides to xyloglucan, which has a naturally high affinity to cellulose. Binding of the chemically modified hemicellulose molecules can thus be used to attach a wide variety of chemical moieties without disruption of the individual fiber or fiber matrix.

Plant fibers have been used for centuries as a versatile raw material for many industries. The main fiber component, cellulose, is composed of long polymers of $\beta(1\rightarrow4)$-linked D-glucose. These glucan polymers adhere to each other by hydrogen bonding and hydrophobic interactions to form crystalline microfibrils. The microfibrils associate with other polymers of the fiber cell walls to form a strong but flexible biocomposite. Plant fibers have low density and excellent load-bearing properties. However, chemical modification of the fiber surfaces for different applied purposes is limited to the hydroxyl groups available on cellulose. Because the integrity of the cellulose microfibril depends on the very same hydroxyl groups, extensive chemical modification gradually destroys the fiber stucture.[1−3] Chemically modified cellulose is thus used in applications which do not rely on the mechanical properties of the microfibrils, such as the superadsorbing hydrogels used in hygiene products.[4] The solvated backbone of cellulose is also exploited as a precursor for chemical modification, leading to other types of materials including cellulose nitrates (i.e., nitrocelluloses), cellulose esters (e.g., cellulose acetate), cellulose ethers (e.g., hydroxyethyl cellulose), and carboxylated derivatives (e.g., carboxymethyl cellulose),[5,6] while continued interest is driving the production of a great number of more exotic derivatives.[5]

In applications which depend on the fiber strength, such as paper or packaging materials, coating or lamination is used instead of chemical modification. In biocomposites, cellulose fibers are blended in a matrix of man-made polymers to achieve lightweight materials with good strength properties.[7] However, because cellulose is a hydrophilic polymer, which is poorly compatible with the typically hydrophobic matrix polymers, such applications suffer from poor interfacial interactions between the natural fibers and the thermoplastic material. This property mismatch leads to decreased performance and limits the amount of reinforcing fibers that can be added. To overcome this problem, direct chemical compatibilization of cellulose has been attempted.[8] However, extensive chemical modification results in loss of fiber structure, and it is only by limiting the degree of substitution of the cellulose hydroxyl groups that the fiber integrity can be maintained.[9]

Xyloglucan is a soluble polymer with a $\beta(1\rightarrow4)$-linked glucan backbone substituted by xylosyl and galactosyl residues, which in turn are sometimes substituted with α-fucosyl residues (Figure 1B). Xyloglucan associates tightly with cellulose microfibrils in the primary cell walls of plants or accumulates as a storage polysaccharide in seeds. Because of their tight binding to cellulose, xyloglucans (as tamarind kernel powder) are used commonly as sizing agents for textiles[10,11] and can also be used as wet-end additives in papermaking.[12,13]

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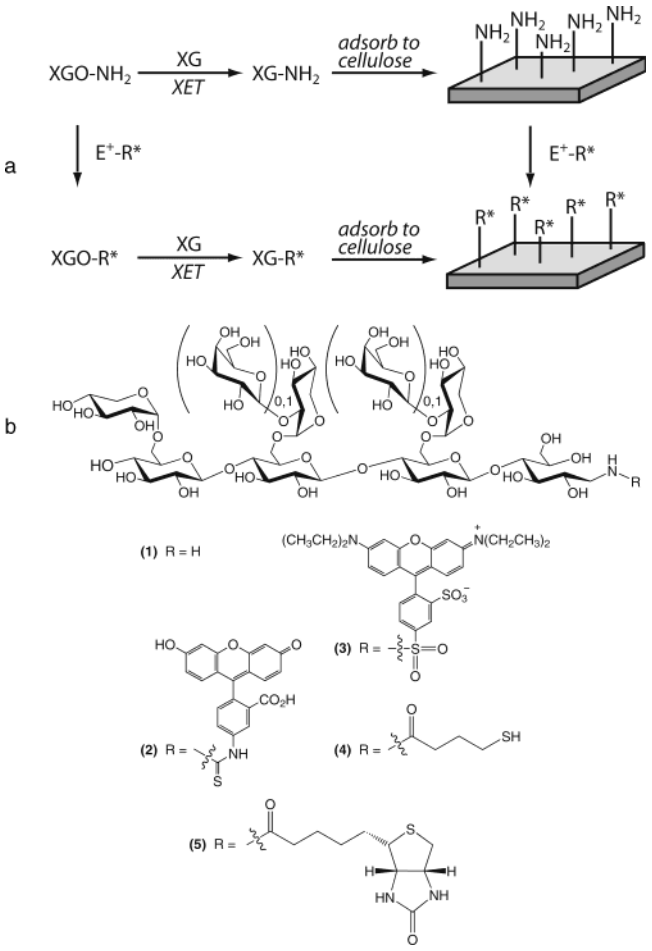

Figure 1. (a) Principles of the topochemical surface modification of cellulosic fibers through enzymatic engineering of xyloglucan. (b) Chemical structures of the aminoalditol xyloglucan oligosaccharide (XGO-NH_2) (**1**) and its derivatives (**2**−**5**) used in this study.

During plant cell growth, the xyloglucan networks cross-linking the cellulose microfibrils must be temporarily loosened and subsequently reinforced.[14] For this purpose, plants produce the enzyme xyloglucan endotransglycosylase (XET). XET is structurally and mechanistically related to microbial glycosyl hydrolases, which carry out carbohydrate degradation. In the first reaction step, both enzymes catalyze the cleavage of a glycosidic bond with subsequent formation of a covalent glycosyl-enzyme intermediate.[15] In the case of the hydrolases, this intermediate is decomposed by water, and the net result is carbohydrate hydrolysis. In contrast, XET typically excludes water as a glycosyl acceptor, but instead transfers the glycosyl moiety to carbohydrate acceptors.[14] Because of this unique transglycosylation mechanism, XET can be exploited for carbohydrate synthesis. In addition to xyloglucan polymers, XET also readily accepts xyloglucan oligosaccharides, even when chemically modified. We have exploited this capacity of XET to introduce different chemically reactive groups to xyloglucan. The xyloglucan polysaccharide carrying new chemically reactive groups can then be bound to different cellulosic surfaces to alter their physicochemical properties. Because the modification is achieved through binding interactions, and not by direct chemical modification of cellulose, the fiber integrity and strength are fully maintained.

Results

Figure 1a shows the principle of using xyloglucan endotransglycosylase (XET), xyloglucan (XG). and chemically modified xyloglucan oligosaccharides (XGO) for the surface modification of cellulose. An aminoalditol derivative of an XGO, XGO-NH_2 (Figure 1b, **1**), serves as the key intermediate, which is incorporated into high M_r xyloglucan using the transglycosylating ability of XET, to produce aminated xyloglucan (XG-NH_2). Following adsorption of the modified xyloglucan to the cellulose surface, the amino group can be specifically derivatized with a number of functional groups (Figure 1a, upper pathway). Alternatively, the reactive XGO-NH_2 may be first derivatized with the final functional group of interest followed by XET-catalyzed incorporation into xyloglucan and subsequent binding to the cellulosic material (Figure 1a, lower pathway). XGOs are ideal starting material because they are small, well-defined molecules and amenable to a variety of controlled chemical transformations. Further, the modified products can be readily characterized by standard methods such as NMR, TLC, and high-resolution MS. In addition to reductive amination,[16,17] XGOs can be derivatized by galactose oxidase oxidation[18] or Königs−Knorr glycosylation (M. Baumann and H. Brumer, unpublished).

Tamarind seed xyloglucan was used as the starting material to produce a mixture of XGOs of XXXG, XLXG, XXLG, and XLLG in the ratio 15:7:32:46, respectively (nomenclature according to Fry et al.,[19] where X represents a Xyl*p*(α1→6)-Glc*p* unit, L represents a Gal*p*(*β*1→2)Xyl*p*(α1→6)Glc*p* unit, and G represents a Glc*p* residue; when written sequentially, a *β*(1→4) linkage between the Glc*p* residues is implied, with the reducing end on the right). Reductive amination of this mixture occurred in good yield to give the corresponding mixture of XGO aminoalditols, XGO-NH_2 (Figure 1b, **1**). This XGO-NH_2 mixture was used directly in the production of amino-modified cellulose surfaces (Figure 1a, upper pathway) or was derivatized further with readily detectable fluorophores, e.g., fluorescein (Figure 1b, **2**) and sulforhodamine (Figure 1b, **3**), *γ*-thiobutyrolactone (Figure 1b, **4**), and biotin (Figure 1b, **5**). These modified XGOs were then used for cellulose surface modification according to the lower pathway of Figure 1a.

Binding Isotherms of Xyloglucan on Cellulose. The high affinity of xyloglucan for cellulose is largely independent of binding temperature, molecular weight, and precise sugar composition of xyloglucan. Further, the binding reaction occurs equally well in water and buffers with different pH and ionic strength.[20−22] To characterize the binding of xyloglucans to cellulosic surfaces, time-course binding experiments of the unmodified polysaccharide were carried out on Whatman No.

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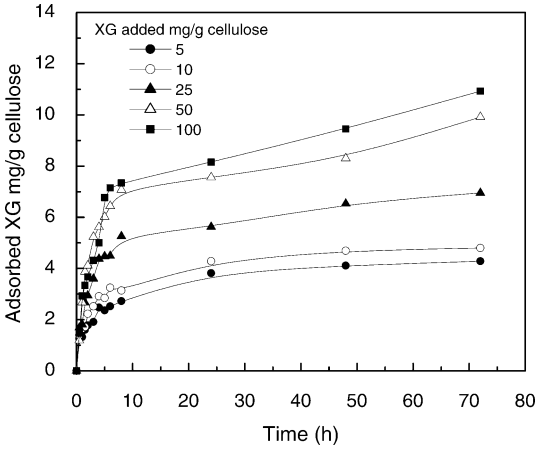

Figure 2. Adsorption isotherms of xyloglucan on Whatman No. 1 filter paper disks.

1 filter paper. With different initial concentrations of xyloglucan, the amount adsorbed onto cellulose from distilled water at 20 °C increased sharply during the first 10 h, more slowly between 10 and 24 h, and continued to creep up even after 24 h of adsorption (Figure 2). This apparent lack of a binding equilibrium is likely to be due to swelling of the cellulose in water, accompanied by a slow increase of its surface area. When the initial amount of xyloglucan was gradually increased from 5 to 50 mg/g cellulose, the final amount of bound xyloglucan increased from 3.8 to 7.5 mg/g during a 24 h incubation. Further increases in the initial amount of xyloglucan did not significantly increase the final bound amount of xyloglucan. Thus, a satisfactory surface coverage was achieved by the addition of 25−50 mg of xyloglucan per gram of cellulose with an adsorption time of about 24 h.

Incorporation of XGO-FITC into XG by XET. To quantitatively monitor the incorporation of chemically functionalized XGOs into xyloglucan during the XET reaction, gel permeation chromatographic (GPC) analysis was performed (Figure 3). The reactions were analyzed directly after lyophilization and dissolution in dimethyl sulfoxide (DMSO) to ascertain the product distribution at each time point. DMSO was chosen as elution solvent to avoid the formation of intermolecular aggregates of xyloglucan.[23] Both evaporative light scattering and fluorescence detectors were used to analyze total and FITC-modified xyloglucans in the column effluent. The sharp peak in each chromatogram at the weight average molecular weight (M_w) of 1300 is due to XGO-FITC as XLLG-, XXLG-, XLXG-, and XXXG-FITC are not resolved under these conditions. The shift in the average M_w of the larger xyloglucan chains from 46.8×10^4 to 1.7×10^4 and the decreasing intensity of the XGO-FITC peak are clearly visible as the reaction proceeds from 0 to 24 h (Figure 3a). Because the fluorescence intensity is directly proportional to the amount of FITC, the GPC curves clearly indicate that the amount of free XGO-FITC decreased with prolonged XET reaction, indicating successful incorporation into the xyloglucan. Interestingly, the production of short

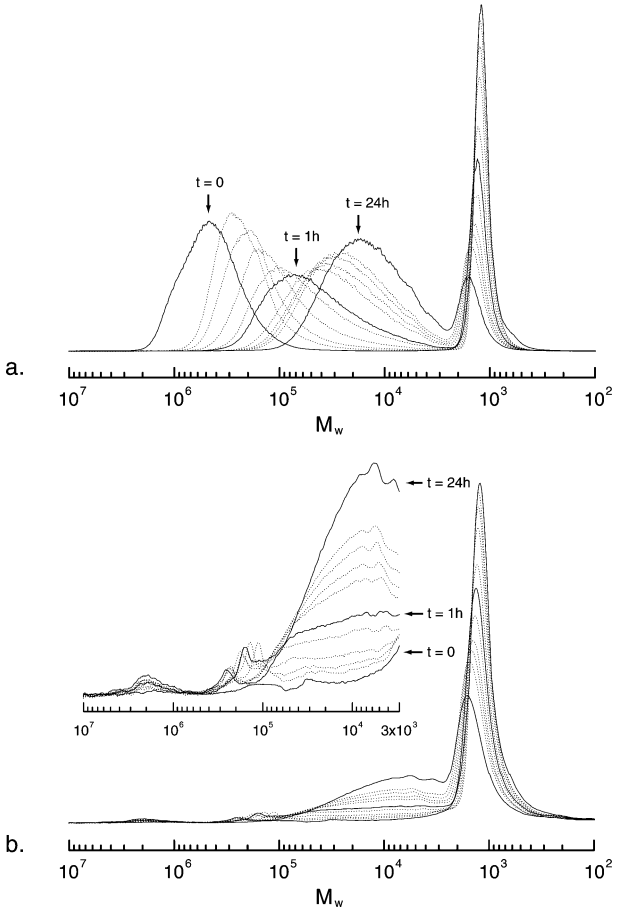

Figure 3. GPC curves for the modified and unmodified xyloglucan produced by XET-catalyzed incorporation of XGO-FITC into XG with increasing time. (a) Evaporative light scattering detection, (b) fluorescence detection.

chain XG-FITC increased with increased reaction time, while the peaks of the longer chain XG-FITC slightly decreased and shifted to lower retention times, indicating the production of higher molecular weight XG-FITC (Figure 3b). Consistent with earlier observations,[24] this suggests that the enzyme chooses its donor substrate independently of its size and attacks it at randomly selected cleavage sites.

Adsorption of FITC-Modified Xyloglucan onto Cellulose. Figure 4a shows the increase in the yellow coloration of Whatman No. 1 filter disks exposed to increasing amounts of XG, which was preincubated with XGO-FITC in the presence of a fixed amount of XET at increasing incubation times (disks B1−B5), and XG preincubated with XGO-FITC in the presence of increasing amounts of XET at a fixed incubation time (disks C1−C5). The increasing bright yellow color in rows B and C visually demonstrates the facile adsorption of the label onto the filter paper surface when XET was used to catalyze the incorporation of XGO-FITC to XG. The control reactions containing only xyloglucan, or XET and XGO-FITC without xyloglucan, failed to color the paper (Figure 4, disks B6 and C6, respectively).

To better quantitate the adsorption of the fluorescein group onto the filter paper surfaces, standard curves were prepared using filter disks carrying known amounts of XG-FITC (Figure

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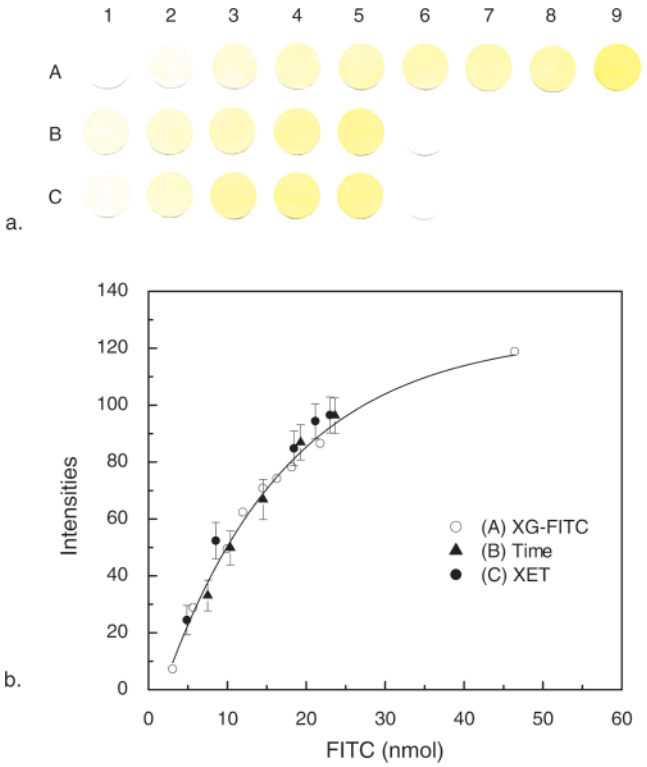

Figure 4. (a) XG-FITC adsorption on Whatman No. 1 fiter paper disks: A1−9, increasing amount of XG-FITC pipetted onto the disks without washing; B1−5, disks adsorbed with XG-FITC produced by 5, 10, 20, 60, and 240 min XET incubation; C1−5, papers adsorbed with XG-FITC produced by incubation with 1.6, 3.2, 9.6, 16.0, and 32.0 units of XET for 40 min; B-6 and C-6, control papers treated under identical conditions but without XET. (b) Relationship between the intensity of the blue channel in full-color RGB mode images and the adsorbed amount of XG-FITC on the surface of the Whatman No. 1 fiter paper disks.

4, disks A1−9). Because recording of the fluorescence emission with a CCD camera suffered from quenching at high XG-FITC loading (manifested as a decrease in signal at XG-FITC amounts higher than 20 nmol), the amount of FITC on the paper surface was quantified by direct imaging with a standard desktop image scanner. When scanning in full-color RGB mode, the intensity of the blue channel was directly proportional to the amount of XG-FITC adsorbed on the paper. The standard curve prepared with XG-FITC gave a good correlation with the intensities calculated from the experimental data obtained for disks B1−B5 and disks C1−C5, indicating that the amount of functional groups adsorbed onto cellulose can be accurately estimated by imaging (Figure 4b).

Surface Analysis. Images obtained by confocal fluorescence microscopy revealed preferential adsorption of XG-FITC to certain sites on the filter paper fiber surfaces (Figure 5a and b), while homogeneous distribution was observed on the regenerated cellulose membrane surfaces (Figure 5c). Inspection of the fluorescence signal distribution on cross sectional confocal scans of regenerated cellulose membranes and regenerated cellulose fibers shows that the binding of XG-FITC and XG-SR is indeed restricted to the surface of each material (Figure 5d−f).

In Situ Modification of Functionalized XG on Cellulose Surfaces. As a second approach, aminated XG (XG-NH_2), prepared by the XET reaction, was adsorbed onto the filter paper surface prior to further derivatization (Figure 1a, upper pathway). The amount of adsorbed amino groups was determined by the

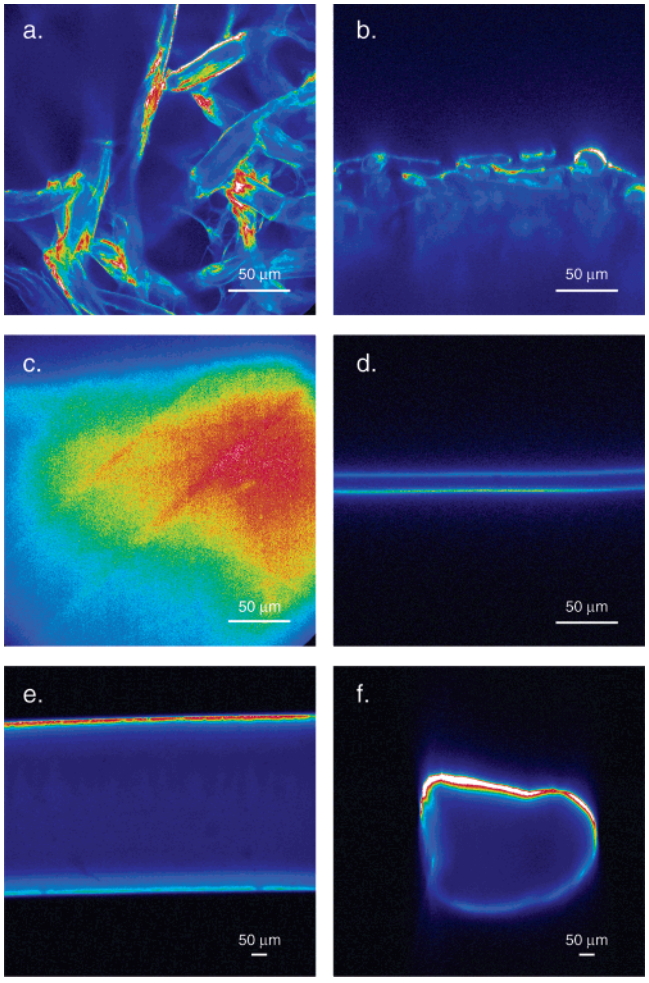

Figure 5. Confocal fluorescence microscopy images of the Whatman No. 1 filter paper disks and the regenerated cellulose membranes and fibers adsorbed with fluorescence-modified xyloglucan produced by XET. (a) Surface image of XG-FITC adsorbed Whatman No. 1 filter paper; (b) confocal cross-sectional image of (a); (c) surface image of XG-FITC adsorbed regenerated cellulose membrane; (d) confocal cross-sectional image of (c); (e) surface image of XG-SR adsorbed regenerated cellulose fiber; (f) cross-sectional image of (e).

fluorescamine assay, which indicated the presence of 0.04 μmol of amino groups per single filter paper disk. The immobilized NH_2 groups were then chemically modified *in situ* by FITC, which resulted in intensive yellow coloration of the modified filter surface (Figure 6a).

Additionally, thiolated xyloglucan (XG-SH, generated by the corporation of **4** into XG by XET) was adsorbed onto the filter paper surface followed by *in situ* reaction with a thiol-specific methanethiosulfonate derivative of rhodamine. In this case, successful fiber surface modification was achieved by disulfide bond formation (Figure 6b). Subsequent treatment with the reducing agent dithiothreitol allowed the fluorophore to be removed by washing. A second treatment with the methanethiosulfonate reagent restored the pink coloration (not shown) and demonstrated that this type of modification can be carried out in a reversible fashion.

In a third example, the XET reaction was thus exploited to generate biotinylated xyloglucan, which was subsequently used for efficient capture of a streptavidin-alkaline phosphatase conjugate onto cellulose surfaces (Figure 6c).

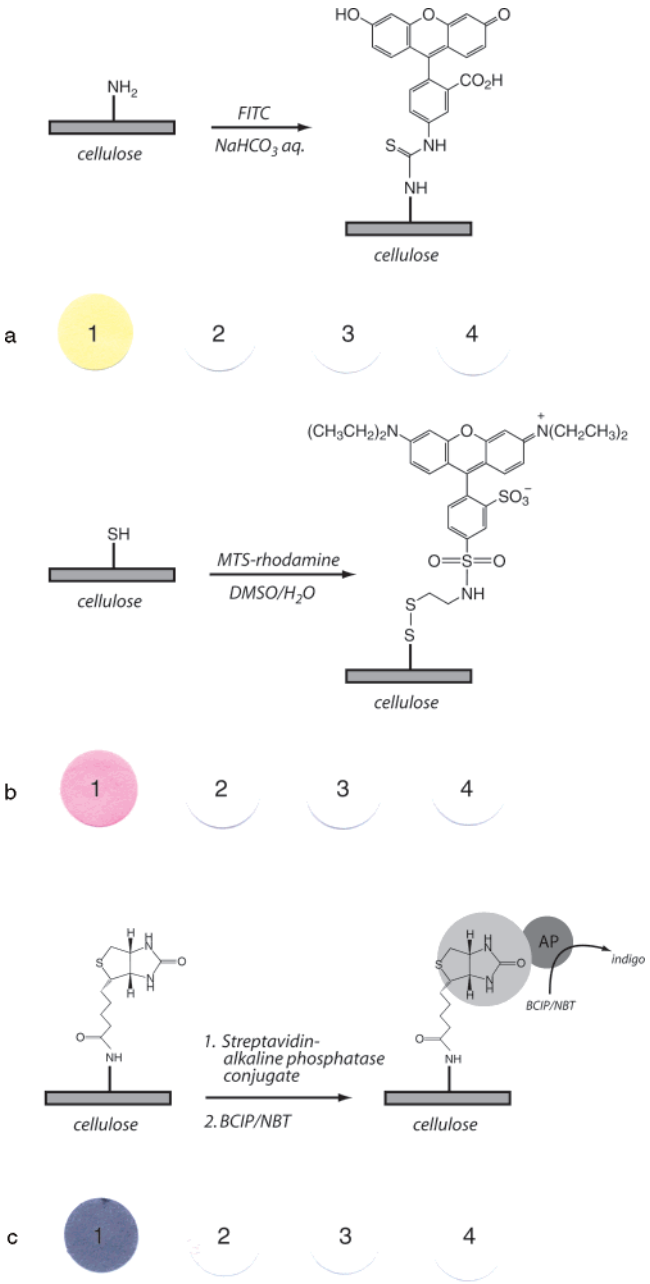

Figure 6. Adsorption of XG-NH_2 (a), XG-SH (b), and XG-Biotin (c) onto Whatman No. 1 filter paper disks and subsequent derivatization with FITC, MTS-rhodamine, or Streptavidin-alkaline phosphatase conjugate/BCIP-NBT, respectively. (1) Paper adsorbed with XG-NH_2, XG-SH, or XG-Biotin; (2) control paper treated with unmodified XG; (3) control paper treated with XGO-NH_2, XGO-SH, or XGO-Biotin; (4) original Whatman No. 1 filter paper.

Conclusion

Xyloglucan has been previously shown to bind to cellulose with high affinity, thereby increasing the paper sheet strength.[12,13] Here, we demonstrate that chemoenzymatic modification of xyloglucan offers an efficient approach to attach new chemical reactivity on cellulose surfaces. The amino group introduced to the xyloglucan is intrinsically more reactive than the hydroxyl groups naturally present in cellulose, which allows the incorporation of a wide range of previously unexplored chemical groups. Because XET operates by first cleaving a long xyloglucan polymer, followed by the attachment of a short chemically modified XGO, the length of the modified xyloglucan, and thus the density of surface modification, can be controlled by choosing appropriate combinations of reaction time and concentrations of the enzyme, xyloglucan, and the modified XGOs. Unlike direct chemical modification of cellulose, the modification through xyloglucan allows the attachment of a high density of the modified groups with no loss of fiber integrity. Because of the modular design, the method is very broad in scope and can thus be used to functionalize cellulose surfaces, for example, to develop novel, high-performance paper and packaging materials, or to design and manufacture advanced biocomposite materials with predefined structures and properties.

Methods

General. Ultrapure water (resistivity ≥ 18.2 MΩ·cm) was used in all experiments. Whatman No. 1 filter paper disks (Ø1.5 cm, average mass 15 mg) were used as a high purity cellulose surface and the starting material for cellulose regeneration. Regenerated cellulose membranes were produced by casting a 6 wt % cellulose cuprammonium solution[25] on a glass plate to give a thickness of 0.2−0.3 mm, followed by coagulation in 10% aqueous NaOH and regeneration in 5% aqueous H_2SO_4, both maintained at 4 °C. The resulting transparent regenerated membranes were washed in running water and dried on a glass plate at 20 °C. Regenerated cellulose fibers were produced by spinning the cellulose cuprammonium solution from a 2 mL syringe, without a needle, directly into the coagulation bath followed by soaking in the regeneration bath. The resulting transparent regenerated fibers were washed with running water and stored in ultrapure water. Xyloglucan from tamarind seed (Xyl:Glc:Gal:Ara = 35:45:16:4) was purchased from Megazyme (Bray, Ireland). A mixture of xyloglucan oligosaccharides (XGO, XXXG/XLXG/XXLG/XLLG[19] ratio 15:7:32:46) was prepared from deoiled tamarind kernel powder (D.N. Palani, Mumbai, India) using endoglucanase digestion.[26] ^1H and ^{13}C NMR spectra were recorded at 298 K in D_2O on a Bruker Avance 500 MHz instrument using a standard 5 mm probe. Electrospray ionization mass spectrometry (ESI-MS) was performed on a Micromass Q-Tof II (Waters Corp., Micromass MS technologies, Manchester, U.K.) in positive ion mode from 1:1 methanol/water containing 0.5 mM NaCl.[27] Negative ion mode spectra were acquired from 1:1 methanol/water containing 0.5% NH_4-OH. External TOF MS calibration using sodium iodide ion clusters was used in both positive and negative ionization modes. Gel permeation chromatography (GPC) measurements were made on a Waters 616 HPLC system equipped with two Tosoh gel columns, G5000HHR and G3000HHR (both 7.8 × 300 mm), connected in series. HPLC-grade dimethyl sulfoxide (DMSO) was used as the eluent at a flow rate of 1 mL/min, and the column temperature was maintained at 60 °C. Analyte detection and quantification were performed both by fluorescence (Shimadzu RF-551, excitation/emission = 480/540 nm) and by evaporative light scattering detection (Polymer Laboratories PL-ELS 1000). Pullulan polysaccharide standards (Polymer Laboratories) were used to calibrate the system over the M_w range 180−788 000.

Xyloglucan Endotransglycosylase (XET). XET was obtained by heterologous expression of the *Populus tremula* x *tremuloides* *Ptt*XET16A[28] in *Pichia pastoris* (Kallas and Teeri et al., in preparation). XET activity was measured using a modification of a published protocol.[29] XET was incubated with a 0.05 mg of XG and 0.05 mg of

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XGO mixture in 100 μL of 20 mM sodium citrate buffer, pH 5.5, for 30 min at 30 °C. The assay was stopped with 0.1 mL of 1 M HCl, and the ionic strength was adjusted by adding 0.8 mL of 20% Na_2SO_4 prior to the addition of 0.2 mL of triiodide solution (0.5% I_2 + 1% KI). Blank assay mixtures contained no XGO mixture. For the purposes of this work, one unit of enzyme activity is defined as that causing a ΔA_{620nm} of 0.1 in 30 min, where $\Delta A_{620nm} = A_{620nm}(\text{blank}) - A_{620nm}(\text{assay})$.

Binding Isotherms of Xyloglucan onto Cellulose. Seven Whatman No. 1 filter paper disks (total mass 0.1 g) were immersed in 5 mL of an aqueous solution containing 0.5, 1, 2.5, 5, and 10 mg of XG in glass vials and incubated at 20 °C with orbital shaking. The XG adsorbed onto the cellulose was measured by the loss of XG from the solution according to the colorimetric method of Kooiman[30] as follows. Next, 50 μL of the solution was withdrawn at various time intervals ranging from 0 to 72 h and mixed with 150 μL of water, 0.8 mL of 20% (w/v) Na_2SO_4, and 0.2 mL of triiodide solution (0.5% I_2 + 1% KI). The amount of bound XG was calculated from the difference in adsorption at 620 nm of the solutions before and after the binding reaction using a standard curve derived from XG solutions of increasing concentrations versus a blank where water replaced the XG solutions.

Chemical synthesis of the XGO aminoalditol (XGO-NH_2, **1**) and the FITC (XGO-FITC, **2**), sulforhodamine (XGO-SR, **3**), sulfydryl (XGO-SH, **4**), and biotin derivatives (XGO-Biotin, **5**) is described in the Supporting Information.

Time Dependence of the XET-Catalyzed Incorporation of XGO-FITC into XG. Samples (200 μL total volume) containing a mixture of XG (1 g/L), XGO-FITC (0.5 g/L), and XET (8 units) in sodium citrate buffer (20 mM, pH 5.5) were incubated at 30 °C for 5, 10, 20, 40, 60, 120, 180, 240, 360, and 1440 min, whereafter the reaction was terminated by heating at 75 °C for 10 min to denature XET. After being cooled to room temperature, the assay solution was centrifuged at 12 000*g* for 20 min at 4 °C to precipitate XET. The supernatant was freeze-dried under vacuum and redissolved in 200 μL of DMSO, and then applied for the GPC analysis. The time zero samples were treated under identical conditions but without XET.

Adsorption of Fluorescein-Modified Xyloglucan (XG-FITC) onto Whatman No. 1 Filter Paper Disks. The reaction conditions for the preparation of XG-FITC were as described above with incubation times of 5, 10, 20, 60, and 240 min. Similarly, a mixture (200 μL total volume) of XG (1 g/L), XGO-FITC (0.5 g/L) in sodium citrate buffer (20 mM, pH 5.5) was incubated with decreasing amounts of XET (32.0, 16.0, 9.6, 3.2, and 1.6 units) at 30 °C for 40 min. At that time, these reactions were treated as described for the time series. The control samples were treated under identical conditions but without XET. After termination of the reactions by heating, each reaction mixture was centrifuged at 12 000*g* for 20 min to remove denatured XET, and the supernatant was withdrawn and diluted to 500 μL with water. A Whatman No. 1 filter paper disk was incubated with each sample in a glass vial under orbital shaking at 20 °C for 24 h. The cellulose disks were then placed into fresh vials, washed repeatedly with water (3 × 5 mL) in an end-over-end mixer, and dried at 60 °C. The amount of the FITC incorporated into XG and subsequently bound to the filter paper was calculated from the loss of XGO-FITC from solution (including the wash solutions), as determined by A_{495nm} in 0.1 M sodium bicarbonate versus a standard curve of XGO-FITC.

Quantification of FITC by Direct Imaging. A standard solution of XG-FITC containing a known amount of FITC was prepared by mixing XG (1 g/L), XGO-FITC (0.5 g/L), and XET (8 units) in sodium citrate buffer (20 mM, pH 5.5, total volume 200 μL). Following incubation at 30 °C for 60 min, the enzymatic reaction was terminated by heating at 75 °C for 10 min. After being cooled to room temperature, 400 μL of ethanol was added and the mixture was centrifuged at 12 000*g* for 20 min at 4 °C to precipitate XG (both modified and unmodified), with the XGO-FITC remaining in the supernatant. The precipitate was dried under vacuum, redissolved in water, and the amount of FITC was determined spectrophotometrically (A_{495}) in 0.1 M sodium bicarbonate versus a standard curve for XGO-FITC. A standard curve for the absorbance of XG-FITC on filter paper was generated as follows. Filter paper disks were shaken with solutions containing 3−50 nmol of XG-FITC on an orbital shaker. Following overnight incubation, the disks were removed and dried in an oven (60 °C). The amount of XG-FITC bound to the paper was quantitated by measuring the remaining amount of XG-FITC in each vial (typically, >95% binding was observed). Filter paper disks were imaged using a standard desktop scanner (ScanJet 6300C, Hewlett-Packard), and the intensity of the blue channel (8 bits of data, intensity values 0−255) from the full color RGB image was extracted using the *Scion Image* software program (version beta 4.0.2, Scion Corp., Frederick, MD).

Adsorption of Fluorescein-Modified Xyloglucan (XG-FITC) and Sulforhodamine-Modified (XG-SR) onto Regenerated Cellulose. Adsorption of XG-FITC onto regenerated cellulose membranes was carried out as for Whatman No. 1 filter disks. A single XG-FITC solution was prepared by a 60 min XET incorporation reaction, as described above. Regenerated cellulose membranes (0.01 g) were immersed in this solution and agitated in an end-over-end mixer for 24 h at 20 °C. The membrane was then removed from the original solution and washed repeatedly with ultrapure water (3 × 5 mL) in an end-over-end mixer, followed by drying on a glass plate at 20 °C. The procedure for the adsorption of XG-SR onto regenerated cellulose fibers was carried out in the same manner by substituting the XGO-SR for XGO-FITC, except that the fibers were stored in ultrapure water without drying.

Confocal Fluorescence Microscopy. Images of cellulosic samples were recorded with a Sarastro 1000 confocal microscope (Molecular Dynamics, Sunnyvale, CA). By using 10/0.32 (dry) and 40/1.0 (oil immersion) objectives, optical section images with thicknesses of approximately 7 and 1 μm were obtained.[31] A wavelength of 488 nm from an argon laser was used for specimen illumination, and fluorescent light at wavelengths longer than 530 nm was detected by a photomultiplier tube. The digital images consist of 512 × 512 pixels, each containing 8 bits of data (light values 0−255). In the figures, pixel intensities are displayed using a pseudocolor scale, where blue/green/yellow/red/white represents an ascending scale of light intensities.

XET Mediated Incorporation of XGO-NH_2 into XG in Solution and Subsequent Adsorption onto Cellulose. A sample containing a mixture (600 μL total volume) of XG (1 g/L), XGO-NH_2 (0.5 g/L), and XET (10 units) in sodium citrate buffer (20 mM, pH 5.5) was incubated at 30 °C for 60 min. Two control samples were used, one containing only XG (1 g/L) and one containing XGO-NH_2 (0.5 g/L) and XET (10 units) but lacking XG. The reaction was terminated, and the denatured XET was removed by centrifugation at 12 000*g* for 20 min. Filter disks were soaked in 500 μL of the supernatant in glass vials and incubated at 20 °C for 24 h with orbital shaking. The cellulose disks were then removed and washed with water (3 × 5 mL) in an end-over-end mixer and dried at 60 °C. The amount of XGO-NH_2 incorporated into XG and subsequently bound to the filter paper was determined by measuring the loss of XGO-NH_2 from solution (including the wash solutions). The XGO-NH_2 concentration was determined using the fluorescamine assay for amino groups essentially as described,[32] except that DMSO was substituted for acetonitrile as the solvent for fluorescamine.

Surface Chemical Modification with FITC. Filter paper disks containing adsorbed XG-NH_2 (0.04 μmol, as determined by fluorescamine assay) were incubated with fluorescein isothiocyanate, isomer I (0.16 mg, 0.4 μmol), in 500 μL of 0.1 M $NaHCO_3$ overnight at room temperature in glass vials with orbital shaking. The filter paper disks

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were washed extensively with 0.1 M $NaHCO_3$ and water and then were dried at 60 °C.

XET Mediated Incorporation of XGO-SH into XG in Solution and Subsequent Adsorption onto Cellulose. This procedure was carried out exactly as described above by substituting the XGO-SH for the aminoalditol derivative, XGO-NH_2. The amount of XGO-SH ultimately incorporated onto the paper surface was not determined, but was assumed to be approximately the same as XGO-NH_2 due to similar enzymatic reaction rates (data not shown).

Surface Chemical Modification with MTS-Rhodamine. Filter disks containing adsorbed XG-SH were incubated with 1 mL of 100 μM sulforhodamine methanethiosulfonate (MTS-rhodamine, catalog no. S699150, Toronto Research Chemicals, Toronto, Canada) in a solution of DMSO/H_2O (1:9) for 20 min at 20 °C with orbital shaking. The paper was washed with DMSO to remove the unreacted MTS-rhodamine, and then with ultrapure water followed by drying at 60 °C.

XET Mediated Incorporation of XGO-Biotin into XG in Solution and Subsequent Adsorption onto Cellulose. This procedure was carried out exactly as described above by substituting the XGO-Biotin for the aminoalditol derivative, XGO-NH_2. The amount of XGO-Biotin ultimately incorportated onto the paper surface was not determined, but was assumed to be approximately the same as XGO-NH_2 due to similar enzymatic reaction rates (data not shown).

Surface Binding with Streptavidin-Alkaline Phosphatase Conjugate. Filter disks containing adsorbed XG-Biotin were incubated with 1 μg of streptavidin-alkaline phosphatase conjugate (Streptavidin-AP, catalog no. S2890, Sigma) in 500 μL of 100 mM tris-HCl buffer (pH = 9.5) for 1 h at 20 °C with orbital shaking. The papers were washed with buffer (3 × 5 mL) in an end-over-end mixer, and then extensively washed by filtration using 100 mL of buffer to remove the unbound Streptavidin-AP. The filters were subsequently incubated with 1 mL of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium alkaline phosphatase substrate (BCIP/NBT, Sigma Fast, catalog no. B-5655, Sigma) for 1 h at 20 °C with orbital shaking. The papers then were treated with 100 mM sodium acetate buffer (pH = 5.5), extensively washed by filtration using 1 L of ultrapure water, and dried at 60 °C.

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Supporting Information Available: Experimental procedures for the synthesis of compounds **1**−**5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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