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Synthesis of Multifunctional Cellulose Nanocrystals for Lectin Recognition and Bacterial Imaging

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Abstract

Multifunctional cellulose nanocrystals have been synthesized and applied as a new type of glyconanomaterial in lectin binding and bacterial imaging. The cellulose nanocrystals were prepared by TEMPO-mediated oxidation and acidic hydrolysis, followed by functionalization with a quinolone fluorophore and carbohydrate ligands. The cellulose nanocrystals were subsequently applied in interaction studies with carbohydrate-binding proteins and in bacterial imaging. The results show that the functional cellulose nanocrystals could selectively recognize the corresponding cognate lectins. In addition, mannosylated nanocrystals were shown to selectively interact with FimH-presenting *E. coli*, as detected by TEM and confocal fluorescence microscopy. These glyconanomaterials provide a new application of cellulose nanocrystals in biorecognition and imaging.

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Notes

The authors declare no competing financial interest.

Supporting Information

Additional characterization data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Cellulose, (1→4)- β -D-glucopyranan, is one of the most ubiquitous renewable polymers on earth, being a major constituent of all plant matter and of many other organisms (e.g., bacteria and tunicates).^{1,2} It was first isolated and described by Anselme Payen in 1838, spurring extensive studies of its structural features, chemico-physical properties, and biosynthesis.^{3–7} Usually, the width of cellulose microfibrils is 2–20 nm, while the length may reach a few tens of micrometers.⁸ By acidic hydrolysis of the biopolymer under controlled conditions,⁹ cellulose nanocrystals (CNCs) can be prepared, where the shape and size depend on its specific origin. For example, CNCs from wood are normally 3–5 nm in width and 100–200 nm in length, while crystals from tunicates are generally larger: 10–20 nm wide and 500–2000 nm long.¹⁰ The many attractive features of CNCs, such as their inherent renewability and sustainability, high strength, large specific surface area, and nanoscale dimension, have led to several applications. CNCs are thus, for example, used for polymer reinforcement,^{11–13} nanocomposite formulations,^{14–16} and tissue engineering scaffolds.¹⁷ For further application, different chemical transformations have been applied to the abundant hydroxyl groups at the surface of the CNCs, including cationization,¹⁸ oxidation,¹⁹ silylation,²⁰ and polymer grafting.²¹ For instance, pH-responsive CNCs have been prepared by ceric-ion-initiated graft polymerization of poly(4-vinylpyridine),²² or modification with dual fluorescent dyes through the thiol–ene reaction.²³ CNCs decorated with aromatic sulfonates or quaternary ammonium groups also displayed multivalent interactions with viruses.^{24,25} In addition, (2,2,6,6-tetramethylpiperidine-1-yl)oxyl (TEMPO)-mediated oxidation of CNCs has been successfully used to chemoselectively transform the primary hydroxyl groups to the carboxylic form, leading to partial or complete solubilization.²⁶ Carboxylated CNCs can subsequently be functionalized with, for example, fluorophores,²⁷ prodrugs,²⁸ and ligands, establishing a promising platform for bioimaging, drug delivery, and other biomedical applications.

It is generally known that protein–carbohydrate interactions play important roles in many physiological processes, including cell–cell recognition, cell–matrix interactions, signal transduction, glycoprotein transport, fertilization, and so on.²⁹ Frequently, these interactions are also involved in pathologies emanating from the adhesion of viruses, bacteria, and parasites to the host cells.³⁰ Thus, a detailed understanding of disease-related carbohydrate–protein interactions is of high importance for therapy development. In this context, carbohydrate-binding proteins, such as lectins, are of special interest. This family of proteins has been identified in all organisms, is of nonimmune origin, and acts through binding to specific carbohydrate structures without showing any enzymatic activities.^{31,32} Furthermore, lectins play pivotal roles in deciphering the glycode through their interactions with glycoproteins, glycolipids, and oligosaccharides.³³ To mimic, study, and apply these interactions, glyconanomaterials have recently emerged as highly useful platforms.^{34–40} Due to their high carbohydrate surface densities, multivalent interactions can be established, leading to high binding affinities compared to the free ligands.^{39,41–44} We have, for example, developed glyconanomaterials based on metal nanoparticles,^{43,45} quantum dots,⁴⁶ carbon nanotubes,⁴⁷ and silica nanoparticles^{48,49} and demonstrated their use in imaging, diagnostics, and therapeutics applications.³⁴ However, the potential cytotoxicities of certain

nanoparticles may present challenges in specific applications.⁵⁰ In this regard, cellulose nanocrystals display low cytotoxicities toward human cells and tissues.^{51,52}

In this study we developed a glyconanomaterial platform based on carboxylated cellulose nanocrystals (C–CNCs) for biorecognition applications involving carbohydrate-based interactions with lectins. The C–CNCs were conjugated with specific carbohydrate ligands, as well as a fluorophore emitting in the yellow-green range, and the binding of the resulting multifunctional C–CNCs was evaluated through the interactions with their corresponding cognate lectins. In addition, the functionalized CNCs were applied to imaging of *E. coli* strains by targeting lectin receptors at the bacterial surface.

EXPERIMENTAL SECTION

Chemicals

Cellulose nanofibers were prepared from commercial sulfite wood pulp provided by Nordic pulp (never-dried, 86% cellulose, 14% hemicelluloses). (2,2,6,6-Tetramethyl-piperidin-1-yl)-oxyl (TEMPO), 4-bromo-1,8-naphthalenedicarboxylic anhydride, HCl (37%), ethylenediamine, *o*-phenylenediamine, bovine serum albumin (BSA), concanavalin A (Con A), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), Müller-Hinton (MH) broth, and sodium hydroxide were purchased from Sigma-Aldrich. Acetic acid, copper(II) sulfate pentahydrate, boron trifluoride diethyl etherate, 10% palladium on carbon (Pd/C), and 1-ethyl-3-(3-(dimethylamino)-propyl) carbodiimide hydrochloride (EDC·HCl) were acquired from Alfa Aesar. Amberlite IR-120 H⁺ resin and *N*-hydroxysuccinimide (NHS) were purchased from Lancaster. *Ricinus communis* agglutinin (RCA120) was from Vector laboratories. Dialysis membranes (Spectra/Por 12000–14000 MWCO) were from Fisher Scientific. Penta-*O*-acetate- α -D-mannopyranoside, penta-*O*-acetate- β -D-galacto-pyranoside, and 2-(2-(2-azidoethoxy)ethoxy)ethanol were prepared according to previous studies.⁵³ Müller-Hinton medium was prepared from MH broth (2.3 g), dissolved in Milli-Q water (100 mL), and sterilized before use. Glutaraldehyde (8%), uranyl acetate, and 200-mesh Cu grids were purchased from Electron Microscopy Sciences. *Escherichia coli* strains ORN 178 and ORN 208 were kindly provided by Professor Paul E. Orndorff (North Carolina State University). BD Falcon 96-well, flat-bottom, microtiter plates were obtained from BD Bioscience.

Instruments

A Vita-Prep3 High-speed blender (Vitamix) was used for cellulose nanofiber preparation. pHs were adjusted using a FE20 FiveEasy pH meter (Mettler-Toledo). Conductometric titration was performed using a SevenCompact conductometer (Mettler-Toledo). X-ray diffraction was carried out with a PW 3040/60 X'Pert Pro diffractometer (Philips). Fourier transform infrared images were measured with a Perkin–Elmer Spectrum 200 FTIR spectrometer equipped with a MTE Golden Gate, single attenuated total reflectance system (Specac). UV absorbance and fluorescence were analyzed using a Cary 300Bio UV absorbance spectrophotometer (Varian) and a Cary Eclipse fluorescence spectrophotometer (Varian), respectively. Microplates were incubated in a microtiter incubator shaker Stuart SI505 (Burlington). Sterilization was carried out using a Tuttnauer EZ9 sterilizer

(Hauppauge). Scanning electron microscopy (SEM) images were obtained using a S-4800 scanning electron microscope (Hitachi). Transmission electron microscopy (TEM) images were obtained using a EM-400T TEM microscope (Phillips) operating at an accelerating voltage of 100 kV.

Preparation of Carboxylated Cellulose Nanofibers

Wood pulp was transformed through TEMPO-mediated oxidation following the protocol described by Saito et al.⁵⁴ Briefly, wood pulp (20 g) suspended in water (2 L) was oxidized with TEMPO (16 mg g⁻¹) and NaClO (10 mmol g⁻¹) at pH 10. The oxidized pulp was thoroughly washed with deionized water by filtration and fibrillated for 5 min with a high-speed blender to prepare a 0.25% (w/w) suspension of individualized nanofibers. Finally, the nanofibers were freeze-dried.

Preparation of C-CNCs

The cellulose nanofibers were converted to C-CNCs using a protocol adapted from Salajková et al.⁵⁵ Nanofibers (5 g) were added to an aqueous HCl solution (3 M, 250 mL), and the resulting suspension was refluxed for 3 h. The suspension was subsequently diluted with distilled water, and the sample was washed by centrifugation (4700 rpm, 20 min) until a turbid supernatant appeared. Finally, the suspension was dialyzed against distilled water until neutral (Scheme S1).

Synthesis of Fluorescent Dye and Carbohydrate Ligands

The fluorescent dye 4-(2-aminoethylamino)-7*H*-benz[de]-benzimidazo[2,1-*a*]isoquinoline-7-one (**R₁-NH₂**),⁵⁶ and two different carbohydrate ligands: 1-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-D-man-nopyranoside (**R₂-NH₂**) and 1-(2-(2-(2-aminoethoxy)ethoxy)ethoxy)-D-galactopyranoside (**R₃-NH₂**), were prepared as previously described.⁵⁷

Dual Functionalization of C-CNCs with Fluorescent Dye and Carbohydrate

EDC·HCl (22 mg, 0.11 mmol) and NHS (13 mg, 0.11 mmol) in water (2 mL) were added into a C-CNC suspension (1 mg/mL, 20 mL). The pH was adjusted to 5 with HCl and was maintained for 30 min to complete the activation of the carboxyl groups on C-CNCs. Solutions of fluorescent dye **R₁-NH₂** (5.0 mg, 0.015 mmol) and mannose derivative **R₂-NH₂** (5.0 mg, 0.016 mmol) in water (2 mL) were added to the C-CNC mixture. The reaction was performed under stirring for 16 h at pH = 7.5–8.5. The product was then dialyzed for 3 days in flowing deionized water to yield **C-CNC-Man**. **C-CNC-Gal** was obtained following the same procedures as for **C-CNC-Man** using galactose derivative **R₃-NH₂** (Scheme 1).

Lectin-C-CNC Binding

C-CNC-Man (2 mg) or **C-CNC-Gal** (2 mg) was incubated in HEPES buffer solution (pH 7.2, 10 mM, 1 mL) containing 3% BSA for 30 min. The samples were centrifuged, and the precipitated nanocrystals were incubated in HEPES buffer (1 mL) without BSA for 20 min, after which the nanocrystals solution was centrifuged again. The precipitate was

immediately treated with HEPES buffer (2 mL) containing Con A or RCA-120 (10 μ g), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (1 mM) and CaCl_2 (1 mM) for 30 min⁴⁹.

***Escherichia coli* Interaction Assays**

E. coli ORN 178 and ORN 208 were cultured separately in sterilized MH medium (25 mL) until an OD_{625} of 0.25 was attained (1×10^7 CFU/mL). The grown *E. coli* cells were centrifuged at 7000 rpm for 10 min, and the precipitation was redispersed in HEPES buffer (3 mL, 10 mM). To *E. coli* (ORN 178 and 208, 100 μ L) in HEPES, **C–CNC–Man** or **C–CNC–Gal** (50 μ L, 0.25 mg/mL) was added in a 96-well plate and incubated in an incubator shaker (250 rpm) at 37 °C for 2 h.

Carboxyl Content Determination

The carboxyl content of C–CNCs was measured by conductometric titration. Freeze-dried C–CNCs (30 mg) were suspended in HCl (40 mL, 0.01 M) and were titrated with NaOH (0.01 M). The degree of oxidation (DO) and carboxyl content of the sample were calculated following the method of da Silva Perez et al.⁵⁸ using eqs 1 and 2:

$$\text{DO} = 162c(V_2 - V_1)[w - 36c(V_2 - V_1)]^{-1} \quad (1)$$

$$c_{\text{content}} = \frac{c(V_2 - V_1)}{w} \quad (2)$$

where c is the concentration of NaOH (0.01 M), V_1 and V_2 are the end point volumes of NaOH (L), and w is the weight of the C–CNCs (Figure S1).

X-ray Diffraction (XRD)

Freeze-dried C–CNCs were pressed on XRD disks, and diffractograms were recorded in reflection mode: 2θ angular 5–40° with a step size of 0.05°. The Cu K α radiation ($\lambda = 1.5418$ Å) produced at the settings of 45 kV and 40 mA was monochromatized by a Ni filter (20 mm). A position sensitive detector was used to record diffractograms from rotating specimens.

Crystallinity Index (CI)

The crystallinity index (CI) was calculated using eq 3:⁵⁹

$$\text{CI} = \frac{I_{200} - I_{\text{am}}}{I_{200}} \times 100 \quad (3)$$

where I_{200} is the intensity of the 200 lattice plane at $2\theta = 22.4^\circ$ and I_{am} represents the peak intensity at 18° , which corresponds to the amorphous material in cellulose (Figure 1A).

Thermogravimetric Analysis (TGA)

Samples were predried under vacuum overnight and then subjected to heating scans from 30 to 800 °C, with a rate of 10 °C/min under a nitrogen atmosphere.

Dynamic Light Scattering (DLS)

DLS was performed at room temperature, using suspensions of C-CNCs, **C-CNC-Man**, or **C-CNC-Gal** (0.05 mg/mL) in HEPES buffer solution (pH 7.2, 10 mM). Samples involving proteins were prepared as indicated for lectin-C-CNC binding (see above), after which the samples were centrifuged and the resulting complexes washed with HEPES buffer to remove surplus lectin. After centrifugation, the **C-CNC-Man**+Con A or **C-CNC-Gal**+RCA120 samples were resuspended in HEPES buffer solution (0.05 mg/mL).

Transmission Electron Microscopy (TEM) and Laser Scanning Confocal Microscopy (LSCM)

E. coli aliquots (20 μ L) incubated with **C-CNC-Man** or **C-CNC-Gal** were added to a 200 mesh Cu grid, dipped in 1% glutaraldehyde solution, and dried overnight. The sample grids were stained using 1% uranyl acetate and observed using TEM and LSCM.

RESULTS AND DISCUSSION

Cellulose Nanocrystals from TEMPO-Oxidized Cellulose Nanofibers

In order to achieve a high degree of carboxylation for further surface modification, C-CNCs were prepared by acid hydrolysis of TEMPO-oxidized cellulose nanofibers (Scheme 1). This route was found to produce higher carboxylate content than the TEMPO-oxidation of cellulose nanocrystals.⁵⁵ Using conductometric titration (Figure S1), the degree of oxidation of the C-CNCs was determined to be 0.27 (eq 1), corresponding to a carboxylate content of 1.56 mmol g⁻¹ (eq 2). The X-ray diffraction patterns exhibited the most intense signal at 22.4°, a wide signal at 15.3°, and a weaker signal at 34.6°, corresponding to the (200), (110), and (004) crystal planes, which are typical for C-CNC crystal structures. The crystallinity index (CI) was estimated to 65% (eq 3; Figure 1A). Scanning transmission electron microscopy (STEM) images displayed C-CNCs with an average length of 265 $\pm$ 80 nm and width of 5.2 $\pm$ 0.3 nm (Figure 1B).

Dual Functionalization of C-CNCs

The C-CNCs were modified with a fluorescent dye (**R₁-NH₂**) that gives yellow-green fluorescence upon excitation at 450 nm and a carbohydrate ligand: either 1-(2-(2-(2-aminoethoxy)ethoxy)-ethoxy-D-mannopyranoside (Man, **R₂-NH₂**) or an analogous β -D-galactopyranoside ligand (Gal, **R₃-NH₂**). The functionalization method involved EDC/NHS-mediated ligation of compound **R₁-NH₂** and mannose derivative **R₂-NH₂** or galactose derivative **R₃-NH₂** to the C-CNCs to yield dually functionalized nanocrystals **C-CNC-Man** and **C-CNC-Gal**, respectively. The suspension of unlabeled C-CNCs was colorless, whereas the functionalized nanocrystals displayed a yellow color under visible light and yellowish green emission upon excitation at 450 nm (Figure S2). The functionalization was also supported by FT-IR spectroscopy. All CNCs showed typical cellulose structural characteristics with O-H, C-H, and C-O stretching vibration absorptions at 3344, 2921, and 1060 cm⁻¹, respectively. After functionalization with dye and ligands, both the carboxylic O-H stretch absorptions at ~3340 cm⁻¹, overlapping with the CNC O-H stretch vibrations, and the C=O absorptions at 1736 cm⁻¹ decreased due to amide formation. In addition, the

increased absorption at 1626 cm^{-1} , overlapping with the bending vibration of absorbed water, supports amide formation (Figure 2).

TGA was carried out to investigate the thermal characteristics of the nanocrystals (Figure 3). Evaporation of water led to the first gradual weight loss. The onset temperatures of C–CNCs, **C–CNC-Man**, and **C–CNC-Gal** were all around $220\text{ }^{\circ}\text{C}$. The largest weight loss occurred at $356\text{ }^{\circ}\text{C}$ for **C–CNC-Man** and **C–CNC-Gal** and $313\text{ }^{\circ}\text{C}$ for C–CNCs, indicating that conjugation of dye and carbohydrate ligands increased the thermal stability of the nanocrystals (Figure S3). In addition, **C–CNC-Man** and **C–CNC-Gal** displayed very similar TGA traces, consistent with analogous conjugation patterns of these materials.

The fluorescence characteristics of the resulting multifunctional nanocrystals were used to estimate the degrees of conjugation of the fluorescent dye and the carbohydrate ligands to the C–CNCs. Dye contents of 0.74 and 0.71 mmol/g for **C–CNC-Man** and **C–CNC-Gal**, respectively, were estimated by comparing the fluorescence intensities of the nanocrystal suspensions with the standard calibration curve of the dye (cf. Figure S4). Since the total carboxyl content of the C–CNCs was 1.56 mmol/g and FT-IR analysis indicated complete conjugation, the ratios of fluorescent dye to carbohydrate could be estimated to $0.91:1$ for **C–CNC-Man** and $0.84:1$ in **C–CNC-Gal**.

C–CNC-Lectin Binding

The biorecognition properties of **C–CNC-Man** were evaluated by incubating the nanocrystals with a specific lectin for α -D-mannopyranosides–concanavalin A (Con A; K_a for D-mannose is $8.2 \times 10^3\text{ M}^{-1}$).⁶⁰ Con A maintains a tetrameric structure at physiological conditions,⁶¹ thus, offering the possibility to participate in cross-linking with the multivalent **C–CNC-Man** nanocrystals and exert aggregation.⁴⁸ Indeed, while the nanocrystal suspension remained dispersed in the absence of Con A (Figure 4AI), when **C–CNC-Man** suspension was incubated with Con A, aggregates formed in 30 min (Figure 4AII). This phenomenon was further supported by TEM. Compared with the unfunctionalized C–CNCs (Figure S5), almost all **C–CNC-Man** nanocrystals formed agglomerates in the presence of Con A (Figure 4B). The **C–CNC-Man** nanocrystals were also subjected to *Ricinus communis* agglutinin I (RCA-120), a tetrameric lectin consisting of two As-sB-type dimers,^{62,63} which specifically recognize galactosyl residues (K_a for galactose is $2.2 \times 10^3\text{ M}^{-1}$),⁶⁴ whereas mannosyl groups show no affinity.⁶⁵ As shown in Figure 4AIII, no dramatic precipitation was observed within 30 min incubation with this lectin. The emission spectra of **C–CNC-Man** suspensions in the absence of lectin or after interaction with lectin were recorded (Figure 4C). The fluorescent intensity of the **C–CNC-Man** suspension incubated with Con A for 30 min was significantly reduced (Figure 4CI, II). In contrast, only a small decrease was recorded when treating **C–CNC-Man** with RCA-120 (Figure 4CIII), likely owing to nonspecific adsorption of RCA-120 to the nanocrystals.

To further evaluate the binding and specificity of the carbohydrate-modified C–CNCs, the biorecognition properties of Gal-conjugated C–CNCs were also studied using Con A and RCA-120, respectively. In this case, while the sample remained dispersed in the absence of

RCA-120 (Figure 5AI) or when incubated with Con A (Figure 5AII), agglomeration occurred in the sample incubated with RCA-120 (Figure 5AIII). The interaction of **C–CNC–Gal** with RCA-120 was further analyzed by TEM (Figure 5B), which showed agglomeration analogous to the **C–CNC–Man**/Con A system due to the multivalent interactions between the carbohydrates and the corresponding lectin. Similarly, the fluorescence intensity of **C–CNC–Gal** after treating with RCA-120 for 30 min showed a drastic decrease (Figure 5CIII). A slight decrease in intensity was observed upon incubating **C–CNC–Gal** with Con A within the same time, again likely owing to nonspecific adsorption (Figure 5CII).

DLS analysis was furthermore performed to monitor the aggregation of the C–CNCs upon lectin binding. Apparent size increases were thus recorded for **C–CNC–Man** in the presence of Con A and for **C–CNC–Gal** with RCA-120 (Figure S6), suggesting that cross-linking occurred between the carbohydrate-functionalized nanocrystals and the specific lectins.

Bacterial Recognition and Imaging—To investigate the potential of the CNCs for bacterial recognition and imaging, the C–CNCs were incubated with two *E. coli* strains: ORN 178 and ORN 208. In order to evaluate materials of a comparable nature, thereby reducing compromising effects from, for example, nonspecific binding, binding studies were performed with the analogous carbohydrate-presenting materials **C–CNC–Man** and **C–CNC–Gal**. *E. coli* ORN 208 is devoid of the fimbrial lectin FimH, selective for α -D-mannosides, whereas ORN 178 expresses the protein at type I pili.⁶⁶ Neither strain expresses any β -D-galactose-selective lectin. From the TEM analysis, it could be observed that a large amount of cellulose nanocrystals were bound to the surface of the bacterial cells when **C–CNC–Man** was incubated with the ORN 178 strain, indicating the specific bacterial recognition of **C–CNC–Man** due to the binding activity of mannose ligands toward the FimH lectin on the pili (Figure 6A). In contrast, almost no nanocrystals were bound to cells of the FimH strain after treating with **C–CNC–Man** (Figure 6B). When **C–CNC–Gal** as incubated with ORN 178 or ORN 208, no nanocrystals were located at the surface of either bacterial strain (Figure S7). These results indicate that carbohydrate ligands attached to CNC surfaces endow the materials with the capacity of selective binding to cognate receptors.

Because **C–CNC–Man** was also functionalized with a fluorescent dye, the interaction between the nanocrystals and the bacteria could be imaged by fluorescence microscopy. As can be seen in Figure 7, fluorescence was observed on ORN 178 samples after incubation with **C–CNC–Man**, and only sporadic fluorescence could be detected in samples treated with the ORN 208 strain. For **C–CNC–Gal**, almost no fluorescence was detected with either ORN 178 or ORN 208 (Figure S8). These results demonstrate that the carbohydrate-functionalized C–CNCs are able to selectively distinguish between bacterial strains. In addition, the fluorescent dye enabled the imaging of the interactions between the nanocrystals and the bacteria.

CONCLUSIONS

In summary, we have synthesized fluorescent cellulose nanocrystals, functionalized with carbohydrate ligands, and used them for carbohydrate-mediated lectin recognition. Oxidation of cellulose nanofibers by TEMPO followed by acid hydrolysis afforded carboxy-

functionalized CNCs, to which both the fluorescent dye and the carbohydrate were conjugated in one step. The biorecognition capabilities of the resulting C–CNCs were assessed from lectin interaction studies, where fluorescence and transmission electron microscopy supported the selective recognition of lectins by the materials. Furthermore, the functionalized C–CNCs were used as bacterial affinity probes, showing selective binding in response to the bacterial surface lectin expression. These studies indicate that cellulose nanocrystals can be efficiently used as nanoplat-forms for lectin recognition and bacterial imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
X-ray diffraction pattern and STEM image of C-CNCs.

Figure 2. Difference FT-IR spectra of C-CNCs, **C-CNC-Man**, and **C-CNC-Gal** (all spectra were baseline-corrected and normalized in relation to the absorption at 1060 cm^{-1}).

Figure 3.
TGA analysis of C-CNCs, C-CNC-Man, and C-CNC-Gal.

Figure 4.

(A) **C-CNC-Man** (I), **C-CNC-Man** treated with Con A (II), **C-CNC-Man** treated with RCA-120 (III) in buffer solution (pH 7.2) under visible (top) and UV (bottom) light. (B) TEM images of **C-CNC-Man** treated with Con A. (C) Emission spectra of **C-CNC-Man** (I) and after incubation with Con A (II) or RCA-120 (III).

Figure 5.

(A) **C-CNC-Gal** (I), **C-CNC-Gal** treated with Con A (II), and **C-CNC-Gal** treated with RCA-120 (III) in buffer solution (pH 7.2) under visible (top) and UV (bottom) light. (B) TEM images of **C-CNC-Gal** after treating with RCA-120. (C) Emission spectra of **C-CNC-Gal** (I) and after incubation with Con A (II) and RCA-120 (III).

Figure 6.
TEM images of *E. coli* ORN178 (A) and ORN208 (B) treated with **C-CNC-Man**.

Figure 7. Confocal fluorescence microscopy images of ORN 178 and ORN 208 incubated with **C-CNC-Man**: bright field images (A, D), fluorescence images (B, E), and merged images (C, F).

Scheme 1.
Synthesis of Carbohydrate-Functionalized, Fluorescent C-CNCs