SUPPORTING INFORMATION

Glycan-Functionalized Fluorescent Chitin Nanocrystals for Biorecognition Applications

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SYNTHESIS AND FUNCTIONALIZATION OF TCNs

**Synthesis of TEMPO-oxidized chitin nanocrystals**

TEMPO-oxidized α-chitin nanocrystals (TCNs) were prepared from shrimp chitin following a previously reported method with a slight modification. Briefly, TEMPO (0.16 g) and NaBr (1 g) were dissolved in distilled water (1 L) at room temperature. Chitin powder (10 g) was then dispersed into the solution under magnetic stirring. The reaction was initiated by the addition of NaClO solution (7.5 mmol/g chitin) dropwise into the dispersion. During the addition of NaClO, the pH of the reaction was maintained at 10 by adding 0.5 M NaOH aqueous solution. After all NaClO was consumed, the reaction suspension was filtered using Whatman Grade No. 1 filter paper. The insoluble solid was washed thoroughly with deionized water until the filtrate was neutral. The solid collected was added to deionized water (500 mL), and was dispersed using a homogenization process with a Microfluidizer M-110EH (Microfluidics Ind., Newton, MA, USA). The chitin suspension was passed 5 times through 200 and 100 µm chambers at a pressure of 1600 bar at room temperature (21 °C), and a stable and transparent aqueous suspension of TCN (1 wt.%) was thus obtained.

**Synthesis of 4-(2-Aminoethylamino)-7H-benz[de]benzimidazo[2,1-a]isoquinoline-7-one (2)**

To a solution of 4-bromo-1,8-naphthalenedicarboxylic anhydride (1 g, 3.6 mmol) in acetic acid (20 mL) was added 4-phenylenediamine (480 mg, 0.44 mmol), and the mixture was refluxed for 6 h. The reaction mixture was extracted with chloroform (20 mL × 2), after which the solvent was evaporated. The crude product was purified by column chromatography on silica gel using dichloromethane as eluent, yielding compound 1 (352 mg, 28%) as a yellow solid. $^1$H-NMR (500 MHz, CDCl$_3$): δ 8.90 (d, $J = 7.5$ Hz, 1H), 8.60 (d, $J = 8.0$ Hz, 1H), 8.54 (d, $J = 4.5$ Hz, 1H), 8.52 (d, $J = 3.5$ Hz, 1H), 8.12 (d, $J = 8.0$ Hz, 1H), 7.49 (m, 2H), 7.89 (m, 2H). $^{13}$C-NMR (125 MHz, CDCl$_3$): δ 115.9, 120.2, 121.3, 122.9, 125.8, 126.0, 127.9, 128.1, 128.6, 131.3, 131.4, 131.6, 131.9, 131.9, 143.9, 148.9, 160.2.

To a solution of compound 1 (138 mg, 0.4 mmol) in diglyme (5 mL) were added copper(II) sulfate pentahydrate (50 mg) and ethylenediamine (240 mg, 4 mmol). The reaction mixture was refluxed for 10 h, after which the solvent was evaporated. The crude product was purified by column chromatography on silica gel using dichloromethane as eluent, yielding compound 2 (43 mg, 32%) as yellow solid. $^1$H-NMR (500 MHz, CDCl$_3$): δ 8.87 (d, $J = 7.5$ Hz, 1H), 8.66 (d, $J = 7.5$ Hz, 1H), 8.61 (t, $J = 7.0$ Hz, 1H), 8.11 (d, $J = 8.5$ Hz, 1H), 7.88 (q, $J = 3.0$ and 3.5 Hz, 1H), 7.70 (t, $J = 8.0$ Hz, 1H), 7.44 (m, 2H), 6.77 (d, $J = 8.5$ Hz, 1H), 3.45 (q, $J = 5.0$ and 6.0 Hz, 2H), 3.20 (t, $J = 6.0$ Hz, 2H). $^{13}$C-NMR (125 MHz, CDCl$_3$): δ 160.9, 150.7, 149.8, 146.9, 143.8, 135.2, 132.1, 128.8, 127.2, 125.1, 124.6, 123.8, 120.9, 120.92, 119.5, 116.0, 110.5, 104.8, 44.8, 40.1. MS (ESI): [M+H]$^+$ calculated for C$_{20}$H$_{16}$N$_4$O: 329.3, found: 329.2.
A solution of penta-O-acetate-α-D-mannopyranoside (300 mg, 0.77 mmol) and 2-(2-azidoethoxy)ethanol (200 mg, 1.1 mmol) in dry dichloromethane (5 mL) was cooled to 0 °C. BF$_3$Et$_2$O (550 mg, 3.8 mmol) was added dropwise, and the solution was stirred at rt overnight. The solution was poured into ice water, extracted with dichloromethane, and the extracts were washed with saturated NaHCO$_3$, brine and dried over MgSO$_4$. The crude product was purified by column chromatography on silica gel using hexane–ethyl acetate (1/1 v/v) as eluent, yielding compound 3 (152 mg, 43%) as a colorless oil. $^1$H-NMR (500 MHz, CDCl$_3$): δ 5.37 (dd, J = 3.5 Hz, 1 H), 5.28 (d, J = 9.5 Hz, 1 H), 5.26 (t, J = 3.0 Hz, 1 H), 4.86 (d, J = 1.0 Hz, 1 H), 4.28 (dd, J = 5.0 Hz, 1 H), 4.10 (d, J = 2.0 Hz, 1 H), 4.07 (m, 1 H), 3.81 (m, 1 H), 3.67 (m, 9 H), 3.65 (m, 3 H), 3.39 (t, J = 5.0 Hz, 2 H), 2.15 (s, 3 H), 2.10 (s, 3 H), 2.04 (s, 3 H), 1.99 (s, 3 H). $^{13}$C-NMR (125 MHz, CDCl$_3$): δ 170.8, 170.1, 170.03, 169.8, 97.8, 70.9, 70.8, 70.2, 70.2, 69.7, 69.2, 68.5, 67.5, 66.3, 62.5, 50.8, 21.0, 20.8, 20.8.

Compound 3 (128 mg, 0.24 mmol) was dissolved in dry methanol (2 mL), and NaOMe (6.8 mg, 0.12 mmol) was added. The reaction mixture was stirred at rt for 1 h. Amberlite IR-120 H$^+$ resin was added to adjust the pH to 7. The mixture was then filtered and the solvent was evaporated to yield compound 4 (92 mg, quant) as a colorless oil. $^1$H-NMR (500 MHz, D$_2$O): δ 4.88 (d, J = 1.5 Hz, 1 H), 3.96 (dd, J = 1.5 Hz, 1 H), 3.88 (m, 2H), 3.82 (m, 1 H), 3.71 (m, 3 H), 3.65 (m, 9 H), 3.50 (t, J = 5.0 Hz, 2 H.). $^{13}$C-NMR (125 MHz, D$_2$O): δ 99.9, 72.7, 70.4, 69.9, 69.6, 69.5, 69.4, 69.2, 66.7, 66.3, 60.9, 50.1.

Compound 4 (82 mg, 0.24 mmol) was dissolved in dry methanol (3 mL), and Pd/C (23 mg) was added. The flask was purged with N$_2$ and filled with H$_2$. The mixture was stirred vigorously at rt for 1 h. The reaction mixture was then filtered through Celite and the solvent was evaporated to yield compound 5 (23 mg, 26%) as a clear oil. $^1$H-NMR (500 MHz, D$_2$O): δ 4.88 (s, 1 H), 3.95 (t, J = 1.5 Hz, 1 H), 3.70 (m, 16H), 2.80 (t, J = 5.5 Hz, 1 H.). $^{13}$C-NMR (125 MHz, D$_2$O): δ 99.9, 72.7, 70.4, 69.9, 69.6, 69.4, 69.3, 66.7, 66.3, 60.9, 47.4. MS (ESI): [M+H]$^+$ calculated for C$_{12}$H$_{25}$NO$_8$: 312.3, found: 312.1.

Synthesis of 1-(2-(2-Aminoethoxy)ethoxy)ethoxy-D-galactopyranoside (8)$^3$

A solution of penta-O-acetate-α-D-mannopyranoside (300 mg, 0.77 mmol) and 2-(2-azidoethoxy)ethanol (200 mg, 1.1 mmol) in dry dichloromethane (5 mL) was cooled to 0 °C. BF$_3$Et$_2$O (550 mg, 3.8 mmol) was added dropwise, and the solution was stirred at rt overnight. The solution was poured into ice water, extracted with dichloromethane, and the extracts were washed with saturated NaHCO$_3$, brine and dried over MgSO$_4$. The crude product was purified by column chromatography on silica gel using hexane–ethyl acetate (1/1 v/v) as eluent, yielding compound 3 (152 mg, 43%) as a colorless oil. $^1$H-NMR (500 MHz, CDCl$_3$): δ 5.37 (dd, J = 3.5 Hz, 1 H), 5.28 (d, J = 9.5 Hz, 1 H), 5.26 (t, J = 3.0 Hz, 1 H), 4.86 (d, J = 1.0 Hz, 1 H), 4.28 (dd, J = 5.0 Hz, 1 H), 4.10 (d, J = 2.0 Hz, 1 H), 4.07 (m, 1 H), 3.81 (m, 1 H), 3.67 (m, 9 H), 3.65 (m, 3 H), 3.39 (t, J = 5.0 Hz, 2 H), 2.15 (s, 3 H), 2.10 (s, 3 H), 2.04 (s, 3 H), 1.99 (s, 3 H). $^{13}$C-NMR (125 MHz, CDCl$_3$): δ 170.8, 170.1, 170.03, 169.8, 97.8, 70.9, 70.8, 70.2, 70.2, 69.7, 69.2, 68.5, 67.5, 66.3, 62.5, 50.8, 21.0, 20.8, 20.8.

Compound 3 (128 mg, 0.24 mmol) was dissolved in dry methanol (2 mL), and NaOMe (6.8 mg, 0.12 mmol) was added. The reaction mixture was stirred at rt for 1 h. Amberlite IR-120 H$^+$ resin was added to adjust the pH to 7. The mixture was then filtered and the solvent was evaporated to yield compound 4 (92 mg, quant) as a colorless oil. $^1$H-NMR (500 MHz, D$_2$O): δ 4.88 (d, J = 1.5 Hz, 1 H), 3.96 (dd, J = 1.5 Hz, 1 H), 3.88 (m, 2H), 3.82 (m, 1 H), 3.71 (m, 3 H), 3.65 (m, 9 H), 3.50 (t, J = 5.0 Hz, 2 H.). $^{13}$C-NMR (125 MHz, D$_2$O): δ 99.9, 72.7, 70.4, 69.9, 69.6, 69.5, 69.4, 69.2, 66.7, 66.3, 60.9, 50.1.

Compound 4 (82 mg, 0.24 mmol) was dissolved in dry methanol (3 mL), and Pd/C (23 mg) was added. The flask was purged with N$_2$ and filled with H$_2$. The mixture was stirred vigorously at rt for 1 h. The reaction mixture was then filtered through Celite and the solvent was evaporated to yield compound 5 (23 mg, 26%) as a clear oil. $^1$H-NMR (500 MHz, D$_2$O): δ 4.88 (s, 1 H), 3.95 (t, J = 1.5 Hz, 1 H), 3.70 (m, 16H), 2.80 (t, J = 5.5 Hz, 1 H.). $^{13}$C-NMR (125 MHz, D$_2$O): δ 99.9, 72.7, 70.4, 69.9, 69.6, 69.4, 69.3, 66.7, 66.3, 60.9, 47.4. MS (ESI): [M+H]$^+$ calculated for C$_{12}$H$_{25}$NO$_8$: 312.3, found: 312.1.
A solution of penta-O-acetate-β-D-galactopyranoside (300 mg, 0.77 mmol) and 2-(2-(azidoethoxy)ethoxy)ethanol (200 mg, 1.1 mmol) in dry dichloromethane (5 mL) was cooled to 0 °C. BF₃·Et₂O (550 mg, 3.8 mmol) was added dropwise, and the solution was stirred at rt overnight. The solution was then poured into ice water, extracted with dichloromethane, and the extracts were washed with saturated NaHCO₃, brine and dried over MgSO₄. The crude product was purified by column chromatography on silica gel using hexane–ethyl acetate (1/1 v/v) as eluent, yielding compound 6 (146 mg, 41%) as a colorless oil. ¹H-NMR (500 MHz, CDCl₃): δ 5.38 (d, J = 3.5 Hz, 1 H), 5.20 (m, 1 H), 5.02 (dd, J = 3.5 Hz, 1 H), 4.57 (d, J = 8.0 Hz, 1 H), 4.14 (m, 1 H), 3.97 (m, 1 H), 3.95 (t, J = 4.0 Hz, 1 H), 3.76 (m, 1 H), 3.66 (m, 9 H), 3.40 (t, J = 5.0 Hz, 2 H), 2.15 (s, 3 H), 2.06 (s, 3 H), 2.04 (s, 3 H), 1.99 (s, 3 H). ¹³C-NMR (125 MHz, CDCl₃): δ 170.4, 170.2, 170.1, 169.4, 101.3, 70.9, 70.7, 70.7, 70.6, 70.4, 70.0, 69.0, 68.8, 67.0, 61.3, 50.7, 20.7, 20.6, 20.6.

Compound 6 (128 mg, 0.24 mmol) was dissolved in dry methanol (2 mL), and NaOMe (6.8 mg, 0.12 mmol) was added. The reaction mixture was stirred at rt for 1 h and Amberlite IR-120 H⁺ resin was added to adjust the pH to 7. The resulting mixture was filtered, and the solvent was evaporated from the filtrate to yield compound 7 (90 mg, quant) as colorless oil. ¹H-NMR (500 MHz, D₂O): δ 4.42 (d, J = 8.0 Hz, 1 H), 4.08 (m, 1 H), 3.91 (d, J = 3.5 Hz, 1 H), 3.77 (m, 11 H), 3.72 (m, 1 H), 3.69 (d, J = 4.5 Hz, 1 H), 3.67 (d, J = 4.5 Hz, 1 H), 3.65 (q, J = 3.5 Hz, 2 H). ¹³C-NMR (125 MHz, D₂O): δ 102.8, 75.1, 72.7, 70.7, 69.7, 69.6, 69.5, 69.4, 69.1, 68.6, 60.9, 50.1.

Compound 7 (82 mg, 0.24 mmol) was dissolved in dry methanol (3 mL), and Pd/C (23 mg) was added. The flask was purged with N₂ and filled with H₂. The mixture was stirred vigorously at rt for 1 h, filtered through Celite, and the filtrate was evaporated to yield compound 8 as a clear oil (53 mg, 71%). ¹H-NMR (500 MHz, D₂O): δ 4.38 (d, J = 8.0 Hz, 1H), 4.05 (m, 1 H), 3.88 (d, J = 3.5 Hz, 1H), 3.70 (m, 12H), 3.55 (t, J = 5.0 Hz, 1H), 3.50 (t, J = 8.0 Hz, 1H), 2.77 (t, J = 5.5 Hz, 2H). ¹³C-NMR (125 MHz, D₂O): δ 102.8, 75.1, 72.7, 70.7, 69.7, 69.6, 69.2, 69.1, 68.6, 60.9, 56.2, 48.8. MS (ESI): [M+H]⁺ calculated for C₁₂H₂₅NO₈: 312.3; found: 312.1.

**Functionalization of TCNs with fluorescent dye and D-mannose**

Deionized water (10 mL) was added to a 50-mL round bottom flask containing TCNs suspension (10 mL), and the solution was stirred at rt. A solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl, 22 mg, 0.11 mmol) and N-hydroxysuccinimide (NHS, 13 mg, 0.11 mmol) in water (2 mL) was added. The pH of the mixture was adjusted to 5.5 and was maintained for 30 min to complete the activation of the carboxy groups on TCNs. The fluorescent dye 2 (15 mg, 0.05 mmol) and mannose derivative 5 (15 mg, 0.05 mmol) were dissolved in water (2 mL) and the solution was added to the TCNs mixture. The reaction proceeded under stirring for 18 hours at pH = 7.5~8.5. The product was then dialyzed for five days in flowing deionized water to yield TCN-dye-Man.

**Functionalization of TCNs with fluorescent dye and D-galactose**

TCN-dye-Gal was prepared following the same procedures as for TCN-dye-Man using galactose derivative 8.
CHARACTERIZATIONS OF TCNS AND FUNCTIONALIZED CHITIN NANOCRYSTALS

Determination of TCN carboxylate content

The carboxylate content was determined by conductometric titration. A homogeneous aqueous suspension of TCNs (0.1% (w/w), 100 mL) was prepared by dilution, and NaCl was added (0.1 mL, 1 M). The pH of the suspension was adjusted to 3.5 with 1 M HCl. After 10 min of stirring, the suspension was titrated with 0.01 M NaOH. The conductivity of the suspension was measured with a conductivity station (SevenCompact Conductivity S230, Mettler-Toledo International Inc., Switzerland), and the pH of the suspension was recorded with a FiveEasy pH meter (FE20, Mettler-Toledo International Inc., Switzerland). The conductivity and pH curves thus obtained reflected the content of both carboxylate and C2 amino groups in the TCNs sample, because the carboxylate groups were apparently indistinguishable from the amino groups in the titration curves. The content of carboxylate and amine groups ($C_{\text{carboxylate+amine}}$) obtained by titration was calculated by the following equation:

$$C_{\text{carboxylate+amine}} = \frac{c \times V}{w}$$

where $V$ is the amount of added NaOH (in L) as shown in Figure S2, $c$ is the concentration of NaOH (mol/L), and $w$ is the weight of oven-dried sample (g). The carboxylate content was obtained by subtracting the content of amino groups from the measured $C_{\text{carboxylate+amine}}$ value (1.22 mmol/g) for the TCNs. As measured by solid-state NMR, the degree of acetylation (DA) of the TCNs was 87%, which corresponds to an amine content of 0.66 mmol/g. Thus the resulting value for the carboxylate content of the TCNs amounted to 0.57 mmol/g.

X-ray diffraction (XRD)

XRD measurements were made on disks prepared by pressing freeze-dried TCNs samples, using a Philips X’Pert Pro diffractometer (model PW 3040/60, Netherlands). Diffractograms were recorded in the reflection mode in a 2$q$ angular range 5–30° by steps of 0.05° at room temperature. The Cu Kα radiation ($\lambda = 1.5418$ Å) generated at 45 kV and 40 mA was monochromatized using a 20 mm Ni filter. Diffractograms were recorded from rotating specimen using a position sensitive detector.

Fourier-transformed infrared spectra of TCNs, TCN-dye-Man and TCN-dye-Gal

Fourier-transformed Infrared (FT-IR) spectra of the TCNs samples were obtained using a Perkin–Elmer Spectrum 200 FTIR (UK) equipped with a MIKII Golden Gate, single attenuated total reflectance (ATR) system (Specac Ltd., London, UK). The ATR crystal was a MKII heated diamond 45° ATR top plate. Samples were prepared by freeze drying aqueous suspensions of TCNs. Figure S3 shows the resulting FT-IR spectra of TCNs, TCN-dye-Man, and TCN-dye-Gal.

Atomic force microscopy

A Nanoscope IIIa Atomic Force Microscopy (AFM, Veeco, Santa Barbara, CA) was used to characterize the morphology and lateral size distribution of the TCN sample. The images were scanned in tapping mode under ambient air conditions (23 °C and 50% relative humidity). RTESP
silica cantilevers (Veeco), each with a spring constant of 40 N/m (values provided by manufacturer) were oscillated at their fundamental resonance frequencies, which ranged between 200 and 400 kHz. Samples were prepared by drying the diluted aqueous suspension of TCN on freshly cleaved mica substrates. The mica was attached to an AFM specimen disk and analyzed. Since the nanocrystals are assumed to be cylindrical in shape, the height of the nanocrystals was taken to be equivalent to the diameter, to compensate for image widening due to the convolution of the tip and the particle. Statistical analysis of length and width of the TCN sample was done with a total number of 500 particles.

**Scanning Transmission Electron Microscopy (STEM)**

The functionalized TCNs and unmodified TCNs were examined by STEM. Drops of dilute TCN suspensions were deposited on holey carbon coated electron microscopy grids. The excess liquid was absorbed with a filter paper, and a drop of negative stain (4% uranyl acetate) was added to the grid, left in contact for 2–3 min, and blotted away before drying the grid at 70% relative humidity. The specimens were examined using a Hitachi S-4800 scanning electron microscope (Japan) equipped with a Hitachi transmitted electron detector operated at 30 kV.

**Lectin-TCN binding analyses**

TCN-dye-Man (1 mL) or TCN-dye-Gal (1 mL) was incubated in HEPES buffer solution (pH = 7.2, 1 mL, 10 mM) containing 3% BSA (10 µL) for 25 min. The sample was immediately treated with HEPES buffer (0.5 mL) containing Con A or SBA (10 µg), MnCl₂·4H₂O (1 mM) and CaCl₂ (1 mM) for 30 min. The resulting mixture was centrifuged at 10000 rpm for 15 min, and the emission spectrum of the supernatant was recorded on a fluorescence spectrophotometer (Varian Cary Eclipse) at an excitation wavelength of 450 nm.
XRD SPECTRUM OF TCNs

Figure S 1 XRD spectrum of unmodified TCNs

CONDUCTOMETRIC TITRATION OF TCNs

Figure S 2 Conductometric titration of unmodified TCNs.
EMISSION SPECTRA OF DYE AND FUNCTIONALIZED TCN

**Figure S 3** Emission spectra of compound 2 (dye), TCN-dye-Man and TCN-dye-Gal. $\lambda_{\text{ex}}=450$ nm, $\lambda_{\text{em}}=512$ nm.

FTIR SPECTRUM OF TCNs

**Figure S 4** FT-IR spectra of unmodified TCNs (pink), TCN-dye-Man (green) and TCN-dye-Gal (grey); arrow indicates the carboxylate region at around 1740 cm$^{-1}$.
NMR, ESI AND EMISSION SPECTRA OF COMPOUNDS 2, 5, 8

**Figure S 4** $^1$H-NMR spectrum of compound 2

**Figure S 5** $^{13}$C-NMR spectrum of compound 2
Figure S 6 ESI spectrum of compound 2

Figure S 7 $^1$H-NMR spectrum of compound 5
Figure S 8 $^{13}$C-NMR spectrum of compound 5

Figure S 9 ESI spectrum of compound 5
Figure S 10 $^1$H-NMR spectrum of compound 8

Figure S 11 $^{13}$C-NMR spectrum of compound 8
**E. coli INTERACTION STUDY**

*E. coli* ORN 178 and ORN 208 (kindly donated by Paul E. Orndorff, North Carolina State University) strains were grown in Mueller Hinton broth until OD625 = 0.2 (1 x 10⁷ CFU/mL). Aliquots (100 µL) of the resulting medium was subsequently transferred into 96-well microtiter plates charged with TCN-dye-Man solution (100 µL/well, 0.5 mg/mL), and incubated at 37 °C at 250 rpm for 2 h in a humidified plate shaker incubator. For TEM images, aliquots (2.5 µL) were transferred onto 200 mesh Cu grids and the excess moisture allowed to evaporate. The grid was subsequently soaked in uranyl acetate solution (4% in PBS) for 5 seconds, fixed with gluteraldehyde (2.5% in PBS) for 2 h, and finally dried *in vacuo* overnight.

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