Functionalized cellulose nanocrystals (CNCs) for cellular labeling and bioimaging

Sebastian Raja[†, ‡], Ahmed E. I. Hamouda[‡], Marcelo A. S. de Toledo[‡], Chaolei Hu[§], Marcela P. Bernardo[†, ‡], Carmen Schalla [‡], Liliane S. F. Leite[†], Eva Miriam Buhl[ǁ], Stephan Dreschers[⊥], Andrij Pich[§], Martin Zenke[‡], Luiz H. C. Mattoso[†], Antonio Sechi[‡,]**

[†]National Nanotechnology Laboratory for Agribusiness (LNNA), Embrapa Instrumentação, São Carlos-SP, 13560-970, Brazil

[‡]Institute of Biomedical Engineering, Dept. of Cell Biology, RWTH Aachen University, Pauwelsstrasse, 30, D-52074 Aachen, Germany

[§]DWI-Leibniz-Institute for Interactive Materials, RWTH Aachen University, Forckenbeckstrasse 50, D-52074 Aachen, Germany

[ǁ]Institute for Pathology, Electron Microscopy Facility, RWTH Aachen University, Pauwelsstrasse, 30, D-52074 Aachen, Germany

[⊥]Klinikfür Kinder- und Jugendmedizin, RWTH Aachen University, Pauwelsstrasse, 30, D-52074 Aachen, Germany

*Email: antonio.sechi@rwth-aachen.de, sebastianrajaorg@gmail.com.

CONTENTS

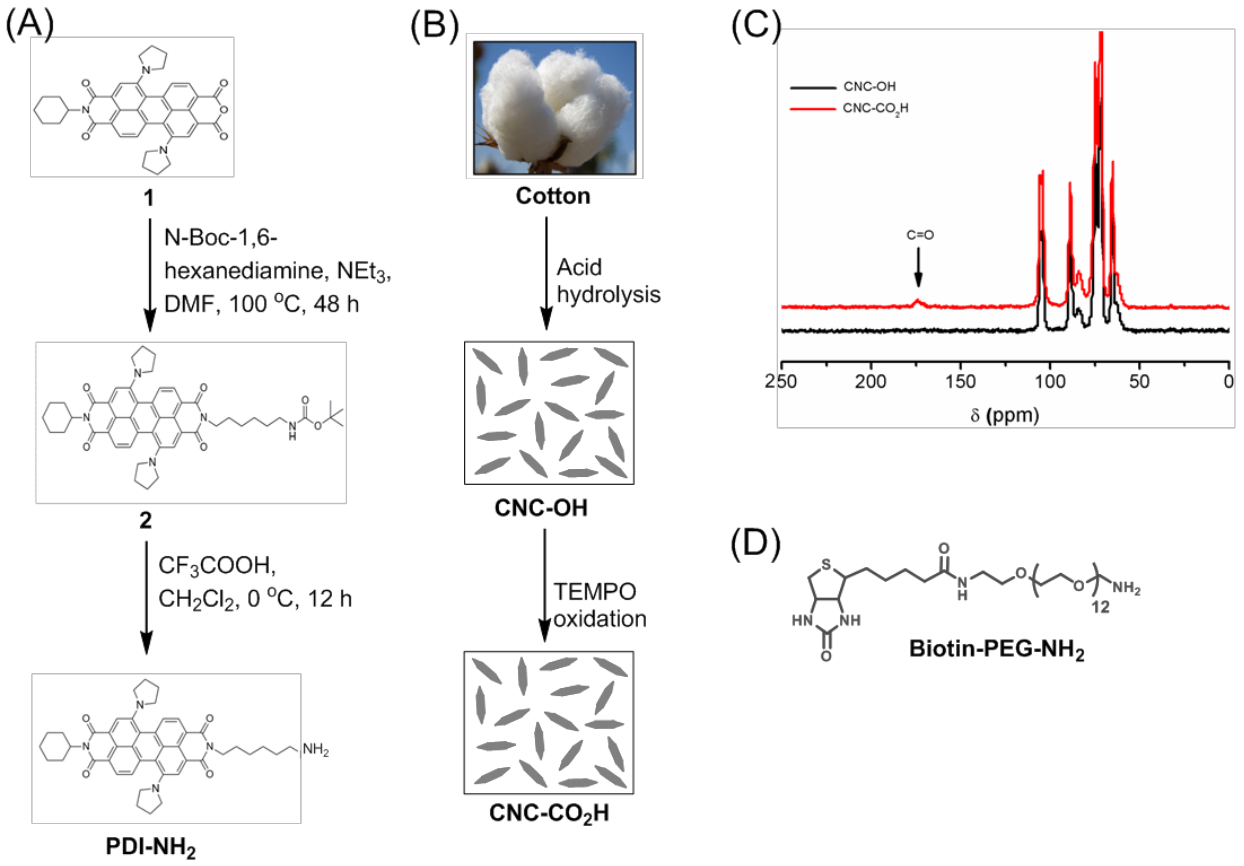

Figure S1. Steps describing the synthesis of the organic fluorophore PDI and the extraction of CNC. (A) Synthesis of perylenediimide (PDI)-based NIR fluorophore. (B) Acid hydrolysis and TEMPO oxidation of cotton linters to generate CNC-OH and CNC-COOH, respectively. (C) Solid-state NMR profile of acid hydrolyzed and TEMPO oxidized CNC. (D) Molecular structure of Biotin-PEG-NH_2.

Scheme S1. Synthetic protocol for perylenediimide (PDI) based fluorophore

Compound 2: Compound **1** was synthesized as described earlier[1]. A mixture of 0.04 g (0.07 mmol) of N-Cyclohexyl-1,7-dipyrrolidinylperylene-3,4:9,10-tetracarboxylic acid-3,4-anhydride-9,10-imide **(1)** and 0.04 g (0.18 mmol) of *N*-Boc-*1,6*-hexanediamine and 0.04 g (0.43 mmol) of NEt_3 in DMF were added into a round-bottom flask. The reaction mixture was stirred in nitrogen atmosphere at 100 °C for 48 h. After completion of the reaction, the reaction mixture was supplemented with dichloromethane (3 x 100 mL) and water (1 x 50 mL). Then, the organic

phase was further subjected to flash column chromatography using 90:10:2 ratio of hexane/acetone/methanol to afford the compounds **2** as greenish solids in 75% yield; ^{1}H NMR (600 MHz, $CDCl_3$): δ1.39-1.42 (m, 16H); 1.71-1.73 (m, 7H); 1.84-1.94 (m, 10H); 2.51-2.57 (m, 2H); 2.66 (m, 6H); 3.60 (m, 4H); 4.11 (t, 2H, 7.2 Hz); 4.97-5.02 (m, 1H); 7.33 (d, 1H, 8Hz); 7.45 (d, 1H, 8Hz); 8.14 (d, 1H; 8Hz); 8.24-8.26 (m, 2H); 8.29 (s, 1H).

Compound 3 (PDI-NH_2): Compound **2** (0.04 g, 0.04 mmol) was dissolved in dichloromethane (6 ml) and TFA (0.25 ml) was added dropwise. The reaction mixture was stirred for 12 hours, at 0 ^{o}C. Then, the reaction mixture was poured into ice and neutralized carefully with NH_3 (solution at 10%) until pH 8. The residue was extracted with dichloromethane, washed with brine, dried over Na_2SO_4 and evaporated to obtain the desired product **3** as a greenish solid in 69% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.39 (m, 7H); 1.70-1.73 (m, 7H); 1.84-1.94 (m, 10H); 2.51-2.57 (m, 2H); 2.66 (m, 6H); 3.60 (m, 4H); 4.11 (t, 2H, 7.2 Hz); 4.97-5.02 (m, 1H); 7.33 (d, 1H, 8Hz); 7.45 (d, 1H, 8Hz); 8.14 (d, 1H; 8Hz); 8.24-8.26 (m, 2H); 8.29 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 13.1, 15.6, 24.5, 24.7, 25.4, 25.6, 25.7, 26.9, 28.1, 28.6, 39.2, 51.1, 51.6, 52.7, 117.1, 117.6, 118.4, 118.6, 119.2, 119.6, 120.3, 120.9, 122.5, 122.7, 124.7, 125.5, 125.6, 127.4, 128.8, 129.7, 130.9, 132.8, 133.1, 139.6, 145.3, 145.5, 160.8, 162.9, 163.0, 163.5.

Equation S1. Determination of the degree of oxidation (DO) by conductometric titration analysis. The carboxyl content of CNC was determined by conductometric titration coupled with pH-metric titration. About 15 mg of CNC was suspended in 200 mL of distilled water and treated by ultrasonic bath for 5 min to remove gas and increase the dispersion. The pH of the suspension is adjusted to acidic condition (pH = 3) with 0.1 M HCl to replace the sodium counter-ions by protons. The suspension was then titrated with 0.2 mL increment of 0.01 M NaOH. The degree of substitution was calculated according to the following equation (1)[2].

$$DO = \frac{162 \times C \times (V_{eq2} - V_{eq1})}{m - 36 \times C \times (V_{eq2} - V_{eq1})} \quad (1)$$

Where 162 ($g.mol^{-1}$) corresponds to the molar mass of an AGU, C ($mol.L^{-1}$) is the concentration of the NaOH solution, m (g) is the weight of the dried sample, 36 in $g.mol^{-1}$ corresponds to the difference between the molecular weight of an AGU (162 $g.mol^{-1}$) and that of the sodium salt of a glucuronic acid moiety (198 $g.mol^{-1}$), and V_{eq1} and V_{eq2} are the equivalent volumes of NaOH on the bends during the titration.

Equation S2. Determination of the degree of oxidation (DO) by solid-state ^{13}C NMR spectroscopy. The degree of oxidation (DO), which corresponds to the number of carboxyl groups per anhydroglucose unit (AGU), was calculated according to the following equation (2):

$$DO = \frac{6 \times I_{168\ ppm}}{I_{Total}} \quad (2)$$

where 6 is total number of carbon peaks for native cellulose, $I_{168\ ppm}$ and I_{Total} are the area intensity of newly obtained carbonyl peak and all the carbon peaks of AGU, respectively.

Equation S3. Determination of the degree of substitution (DS) by elemental analysis. The degree of substitution (DS), which corresponds to the number of grafting groups per AGU, was calculated using the following equation (3)[3]:

$$DS = \frac{N\% \times M_{AGU}}{14n - (N\% \times M_{grafting})} \quad (3)$$

where N% is weight percentage of nitrogen content in the CNC. M_{AGU} and $M_{grafting}$ are the molecular weight of AGU and grafting moiety, respectively. *n* is the number of nitrogen atoms in the grafting group.

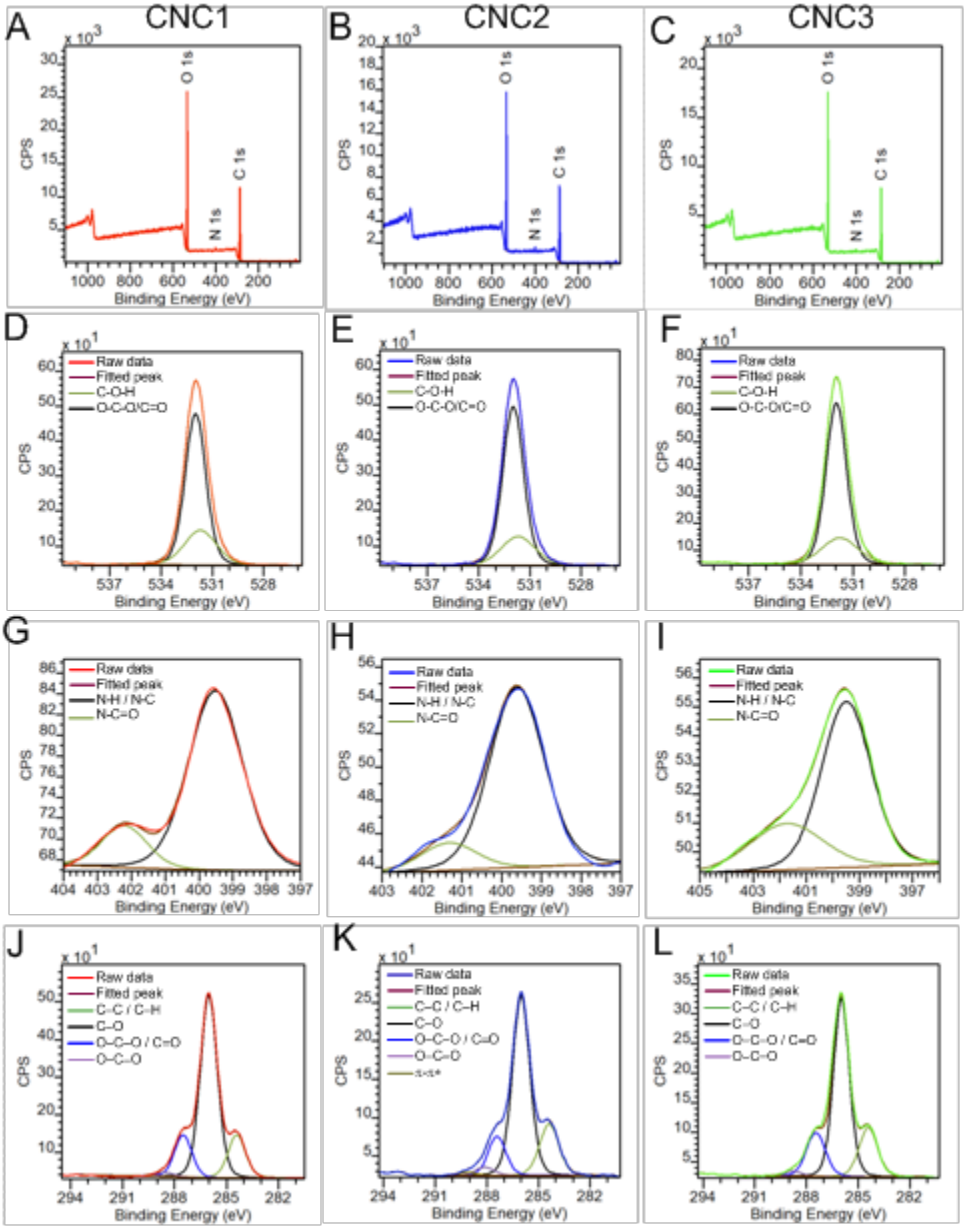

Figure S2. Surface composition of modified CNCs. (A, B and C) XPS survey spectrum of CNC**1**, CNC2 and CNC**3**. Representative high-resolution spectra of O 1s, N 1s and C 1s for CNC**1** (D, G and J), CNC**2** (E, H and K) and CNC**3** (F, I and L), respectively.

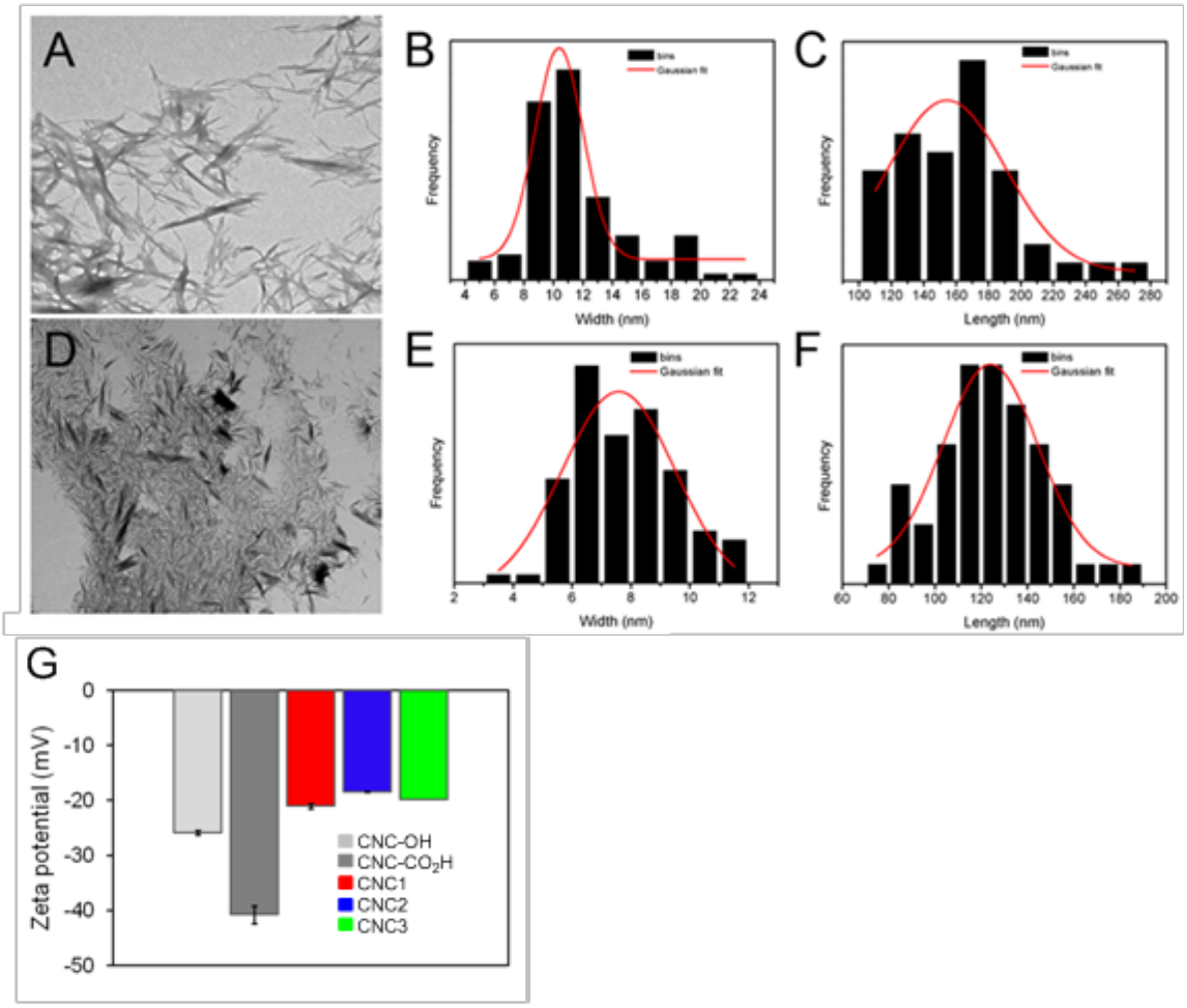

Figure S3. Morphology, size and surface charge characterizations of CNC-OH and CNC-CO_2H. (A-C) TEM images and their respective size distributions (width and length) of CNC-OH. (D-F) TEM images and their respective size distributions (width and length) of CNC-CO_2H. (G) Zeta potential profile of CNCs. Graph shows means ± standard deviations (n = 3).

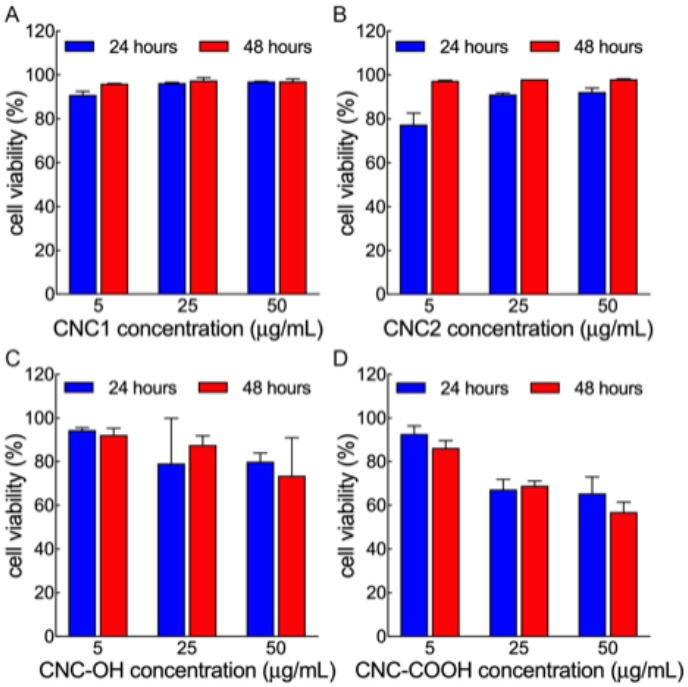

Figure S4. Analysis of dendritic cell viability after exposure to CNCs. Murine DC cells incubated with CNC**1** (A), CNC**2** (B), CNC-OH (C) or CNC-COOH(D) at different concentrations (5 to 50 µg/mL) for 24 h or 48 h at 37 °C. Histograms show mean ± standard deviation (n = 4).

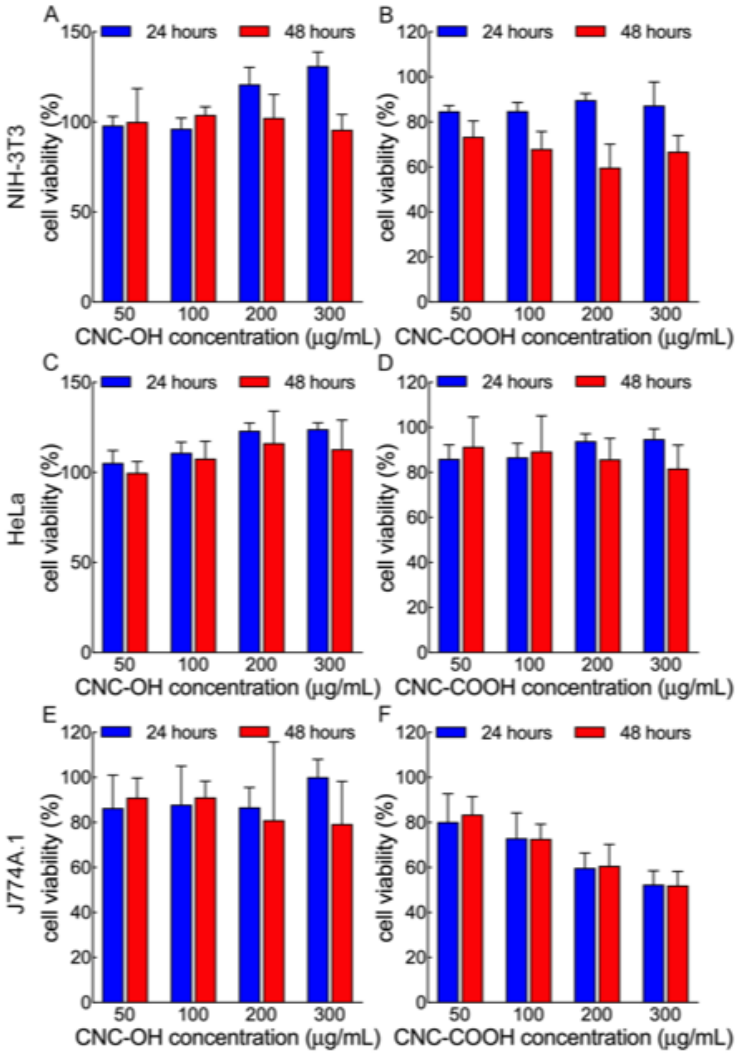

Figure S5. Biocompatibility of precursors CNCs. Assessment of cell viability of NIH-3T3 (A-B), HeLa (C-D) and J774A.1 (E-F) cells incubated with CNC-OH or CNC-COOH at different concentrations (0 to 300 µg/mL) for 24 h or 48 h at 37 °C. Histograms show mean ± standard error (n = 4).

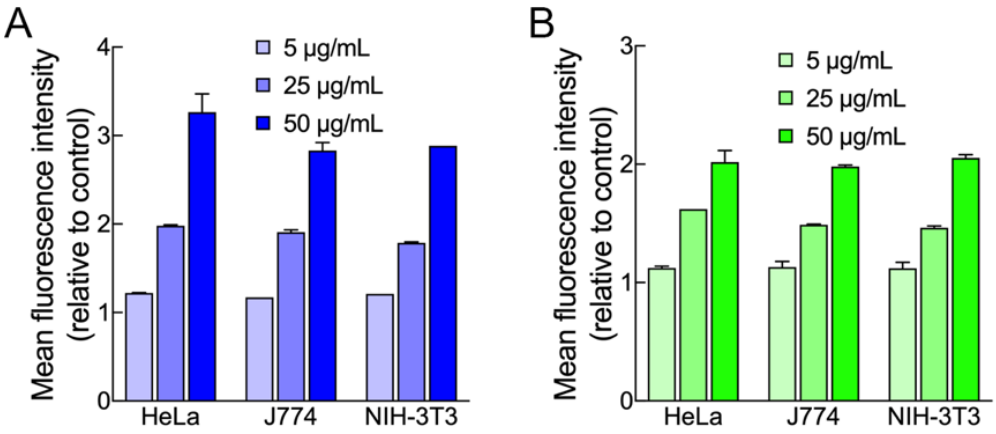

Figure S6. FACS analysis of cells incubated with CNC**2** or CNC**3**. HeLa, J774A.1 and NIH-3T3 were incubated with various concentrations ofCNC**2** or CNC**3** for 24 hours at 37 °C and then analyzed by fluorescence activated cell sorting. Quantification of the mean fluorescence intensity of CNC**2** (A) and CNC**3** (B) from three independent experiments. Note the correlation between the fluorescence signal (PE-Cy7 channel; excitation: 496 nm; emission 785 nm) of CNCs and their concentration. Graphs show mean ± s.e.m (n = 3).

Table S1. Physico-chemical parameters of CNCs

Sample	%C (corrected)[a]	%H[a]	%N[a, d]	DO	DS	length (nm)[e]	width (nm)[e]	ZP (nm)[f]
CNC-OH	43.63 (44.44)	6.25	0.16		-	154 ± 36	10.3 ± 1.6	-25.8
CNC-CO_2H	49.02 (52.18)	5.50	0.46	0.10[b]	0.14[c]	123 ± 20	7.5 ± 1.9	-40.7
CNC**1**	59.54 (62.70)	5.77	1.71 (1.25)	-	0.03[d]	148 ± 20	10.2 ± 1.8	-21.0
CNC**2**	69.63 (72.79)	3.72	4.18 (3.72)	-	0.13[d]	155 ± 40	13.0 ± 3.0	-18.4
CNC**3**	75.73 (78.89)	3.85	3.07 (2.61)	-	0.04[d]	143 ± 13	10.0 ± 2.3	-19.8

[a]The elemental composition of all CNC (corrected with water content) was determined by elemental analysis. [b]Degree of oxidation of CNC was determined by conductometric titration analysis. [c]Degree of oxidation of CNCs was determined by solid-state NMR spectroscopy. [d]Degree of substitution (DS) of modified CNCs was determined based on the N content (corrected with unmodified CNCs and the values are presented in the bracket) from the elemental analysis. [e]CNC lengths and widths were obtained from TEM images (histograms shown in Figure 3 and S3 (A-F)). [f]The zeta (ζ) potential was obtained from DLS measurements (Figure S3G). Values for all measurements are means of 3 replicate samples, except for TEM, where n > 80 particles were measured. Errors represent standard deviations.

Table S2. XPS analysis of modified CNCs

	Surface composition (%)			
Sample	**O 1s (BE)**	**C 1s (BE)**	**N 1s (BE)**	**O/C**
CNC**1**	38.19 (533 eV)	61.08 (286 eV)	0.92 (400 eV)	0. 62
CNC**2**	37.30 (533 eV)	61.44 (286 eV)	1.25 (400 eV)	0. 60
CNC**3**	36.72 (533 eV)	61.79 (286 eV)	1.54 (400 eV)	0. 59

Table S3. Statistical analysis for the viability of HeLa, J774 and NIH-3T3 cells exposed for 24 hours to CNC**1**, CNC**2** or CNC**3**. All CNC concentrations were compared to untreated control cells.

	CNC**1**			CNC**2**			CNC**3**		
CNC (μg)	HeLa	J774	NIH-3T3	HeLa	J774	NIH-3T3	HeLa	J774	NIH-3T3
50	0.0003	0.6374	0.6024	0.6052	<0.0001	0.0002	0.0004	<0.0001	0.0011
100	<0.0001	0.0104	0.0203	0.3965	<0.0001	<0.0001	0.0025	<0.0001	0.0003
200	<0.0001	0.0028	0.0049	0.5487	0.0001	0.0006	<0.0001	<0.0001	0.0015
300	0.0005	0.0024	0.0227	0.6449	0.0004	0.0012	0.0005	<0.0001	0.0503

Table S4. Statistical analysis for the viability of HeLa, J774 and NIH-3T3 cells exposed for 48 hours to CNC**1**, CNC**2** or CNC**3**. All CNC concentrations were compared to untreated control cells.

	CNC**1**			CNC**2**			CNC**3**		
CNC (μg)	HeLa	J774A.1	NIH-3T3	HeLa	J774A.1	NIH-3T3	HeLa	J774A.1	NIH-3T3
50	<0.0001	<0.0001	0.1268	<0.0001	<0.0001	<0.0001	<0.0001	0.0004	<0.0001
100	<0.0001	<0.0001	0.0011	<0.0001	<0.0001	<0.0001	<0.0001	0.0004	<0.0001
200	<0.0001	<0.0001	0.0012	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	<0.0001
300	<0.0001	<0.0001	0.0003	<0.0001	<0.0001	<0.0001	<0.0001	0.0002	0.0110

Table S5. Statistical analysis for the viability of HeLa, J774A.1 and NIH-3T3 cells exposed for 24 hours to CNC-OH or CNC-COOH. All CNC concentrations were compared to untreated control cells.

	CNC-OH			CNC-COOH		
CNC (μg)	HeLa	J774A.1	NIH-3T3	HeLa	J774A.1	NIH-3T3
50	0.0164	0.9489	0.5835	0.0204	0.0025	0.0004
100	0.1402	0.0579	0.8541	0.2563	0.0010	0.0069
200	0.0719	0.0077	0.7135	0.2726	0.0470	0.0409
300	0.1838	0.9930	0.7230	0.0352	0.0094	0.0047

Table S6. Statistical analysis for the viability of HeLa, J774A.1 and NIH-3T3 cells exposed for 48 hours to CNC-OH or CNC-COOH. All CNC concentrations were compared to untreated control cells.

	CNC-OH			CNC-COOH		
CNC (μg)	HeLa	J774A.1	NIH-3T3	HeLa	J774.A1	NIH-3T3
50	0.0047	0.7840	0.6435	0.0241	0.0060	<0.0001
100	0.1927	0.9999	0.6235	0.8331	0.0016	<0.0001
200	0.9519	0.4199	0.0297	0.0005	0.0373	0.0004
300	0.9777	0.0030	0.9880	0.0002	0.0191	0.0001

Table S7. Statistical analysis for the viability of dendritic cells exposed for 24 hours to CNC**1**, CNC**2**, CNC-OH or CNC-COOH. All CNC concentrations were compared to untreated control cells.

CNC (μg)	CNC**1**	CNC**2**	CNC-OH	CNC-COOH
5	0.0001	0.0004	0.8088	0.1242
25	0.0069	0.0280	0.0414	<0.0001
50	0.0147	0.0458	0.0501	<0.0001

Table S8. Statistical analysis for the viability of dendritic cells exposed for 48 hours to CNC**1**, CNC**2**, CNC-OH or CNC-COOH. All CNC concentrations were compared to untreated control cells.

CNC (μg)	CNC**1**	CNC**2**	CNC-OH	CNC-COOH
5	0.0039	0.0004	0.4983	<0.0001
25	0.0285	0.0280	0.1765	<0.0001
50	0.0160	0.0458	0.0038	<0.0001

References

1. Würthner, F.; Stepanenko, V.; Chen, Z.; Saha-Möller, C. R.; Kocher, N.; Stalke, D. Preparation and Characterization of Regioisomerically Pure 1,7-Disubstituted Perylene Bisimide Dyes. *J. Org. Chem.* **2004,** *69,* 7933–7939.
2. Habibi, Y.; Chanzy, H.; Vignon, M. R. TEMPO-Mediated Surface Oxidation of Cellulose Whiskers. *Cellulose* **2006,** *13,* 679–687.

3. Huang, J.; Li, C.; Gray. D. G. Cellulose Nanocrystals Incorporating Fluorescent Methylcoumarin Groups. *ACS Sustainable Chem. Eng.* **2013,** *1*, 1160–1164.