Supporting Information

Cellulose Nano Crystals as effective in inhibiting host cell bacterial adhesion.

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CNCs extraction and characterization

Procedure S1: Acidic hydrolysis

CNCs were extracted from Whatman#1 filter paper by acid hydrolysis in 64% H_2SO_4 for 1 h at 55°C at acid/cellulose ratio of 10 w/w. After acidic treatment, the content of the flask was poured into 10-fold volume of cold water and stirred. Cellulose sediment was purified by repeated cycles of centrifugation at the acceleration of 3200 g for 15 min, and the resuspension of the solid component with distilled water was carried out by ultrasonic mixing for 5 min. After a turbid supernatant was obtained, the suspension was dialyzed against distilled and then ultrapure Milli-Q water for 1 week until pH of 6 was reached.

DLS

DLS and ζ-potential measurements were performed using a Malvern Zetasizer Nano ZS90. The zeta potential data were processed by Zetasizer Software. The samples were prepared by dilution of the initial particles solution (8 g/L) in Milli-Q water obtaining the final concentration of 80 mg/L. Measurements were carried out at 25°C using a disposable cuvette with 10 mm optical path length. Three replicate measurements per sample were performed to establish measurements repeatability. We found a ζ-potential of −39.0 ± 1.0 mV, a dimension of 92.4 ± 1.8 nm (before functionalization), a ζ-potential of −40.6 ± 1.3 mV, and a dimension of 105.0 ± 6.1 after functionalization.

AFM

For AFM analysis, 500 µL of sample of the CNCs was diluted with 1000 µL of distilled water, and 50 µL was immediately added onto freshly cleaved mica at room temperature for 5 min: samples were washed and dried under gentle nitrogen flow. AFM analysis was carried out on a Multimode AFM with a Nanoscope V system (Veeco/Digital Instruments, Mannheim, Germany) operating in tapping mode, using standard antimony(n) doped silicon probes (T, 3.5−4.5µm; L, 115−135µm; K,20−80 N/m) (Bruker Corporation, Billerica, MA) with a scan rate in the 0.5−1.2 Hz range proportionally to the area scanned, and the images were acquired in height and amplitude error. AFM images were analyzed by Scanning Probe Image Processor (SPIP-version-5.1.6, release April 13, 2011) data analysis package. To exclude the interference of possible artifacts, extra control samples, such as freshly cleaved mica and freshly clave mica soaked with buffer, were also used. All the topographic patterns and SPIP characterization described were repeated by additional measurements on a minimum of five different, well-separated areas.

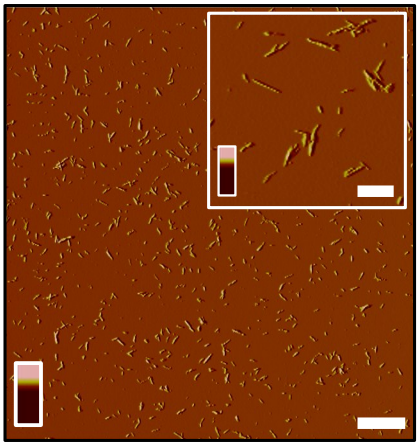

Figure S1: Representative tapping mode of AFM images as determined by height data of CNCs (Z range: −5/+20 nm). Scale bar, 2 µm; inset, 100 nm.

TEM

Transmission Electron Microscopy (TEM) was used to investigate the structure of the isolated cellulose nanocrystals. 10 μL of the nanocrystals preparation was dropped onto Formvar®-coated 300 mesh copper grids and after 5 min the solution was gently removed. Samples were counterstained for 5 min with a saturated solution of uranyl acetate, washed with MilliQ water to eliminate excess uranyl acetate, and allowed to air dry. TEM analyses were performed on a Zeiss LEO 912ab Energy Filtering TEM operating at 120 kV, and images were collected using a CCD-BM/1K system. Digital images were taken at a magnification of 15k.

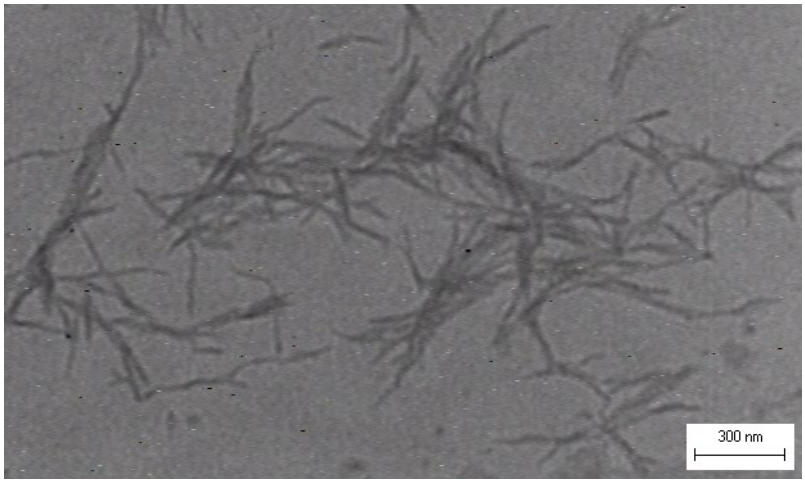

Figure S2: TEM analysis of extracted CNCs

Procedure S2: Conductometric Titration

Titrations were done with 50 ml portions of 0.17% w/v CNC suspensions not neutralized and fresh dialyzed against water MilliQ, in order to assure the removal of all the dissolved ions. NaCl was added to reach a concentration of 1 mM. Then the CNC suspension was placed in a 100 mL round-bottom flask and the conductivity electrode were inserted (Conductivity meter 160, AMEL Instruments). The suspension was titrated under nitrogen with a 0.02 M sodium hydroxide solution which was fresh standardized with potassium hydrogen phthalate. The conductivity values were recorded during the titration. The amount of half-ester sulfate in mmol/g was calculated from the titration results as:

$$R - OSO_3H\left(\frac{mmol}{g}\right) = \frac{[NaOH]x\,V_{NaOH}}{[CNC]x\,V_{CNC}}\,x\,100$$

where [NaOH] was the concentration of the titrant (mol/L), V_{NaOH} was the titrant volume at the equivalence point (mL), [CNC] is the concentration of the CNC suspension (expressed in g/100 mL) and V_{CNC} is the amount of CNC suspension titrated (mL). The data reported is the average of three experiments.

Procedure S3: Alexa labelling

To a suspension of CNCs (0.8%, 20 mL), 1 mg of Alexa Fluor 633 hydrazide, bis-(triethylammonium) salt, and 200 µL of acetic acid were added. The mixture was left to react at room temperature in the dark. After 24 h, the mixture was dialyzed against distilled water for 1 week to remove the acetic acid and the unreacted fluorophore. The UV-Vis absorption spectra were collected with a UV-Vis spectrophotometer Evolution 300 (Thermo Scientific).

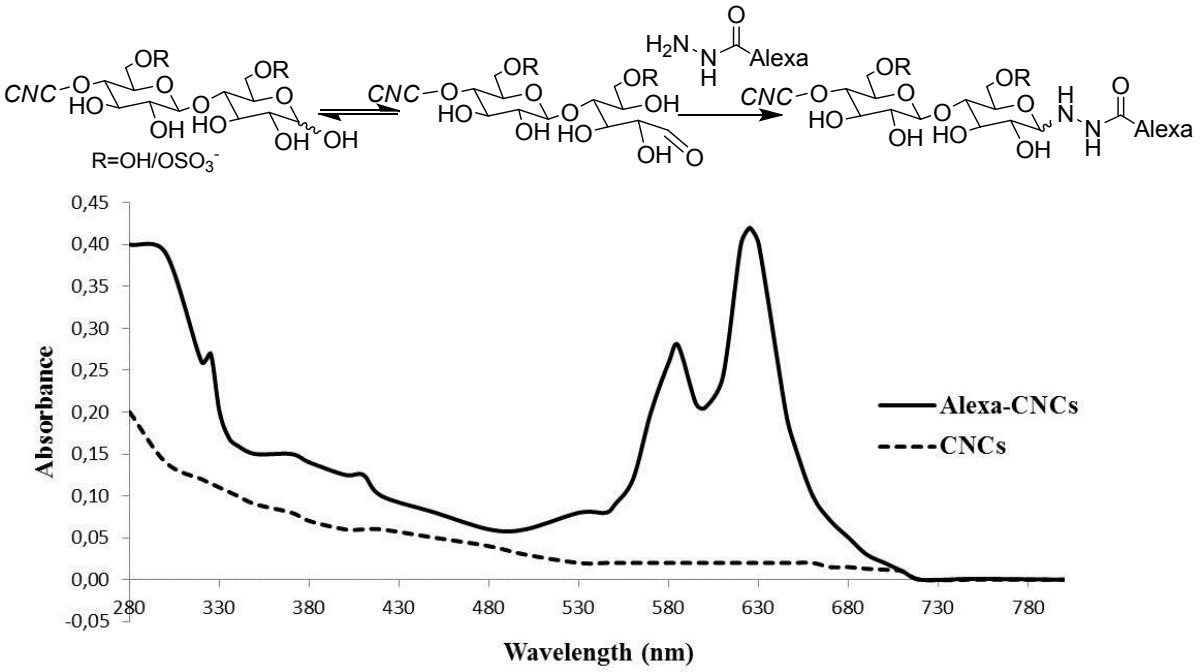

Figure S3: Formation of the CNCs-Alexa Fluor® 633 hydrazine conjugates (upper panel). UV-Vis spectra of CNCs before and after modification with Alexa Fluor® 633 (lower panel).

Fluorescent microscopy

Covered glass bottom agarized were used to immobilize *E. coli* in the presence and absence of CNCs at the concentration of 0.1 %. The CNCs were stained with Alexa Fluor 633 (Ex/Em of the conjugate: 624/643 nm); and bacteria were stained with SYBR® Safe DNA Gel Stain (Ex/Em of the conjugate: 497/520 nm). The stained CNCs and stained bacteria were analyzed with a Fluo View FV100 (Olympus) Fluorescence Microscopy.

Bacterial survival and growth with CNCs

The minimum inhibitory concentration of the CNCs against *E. coli* ATCC 25922 was determined using a dilution method and by subculturing the test dilutions on TSA agar plates after incubation for 24h. The concentration at which there was no growth on agar plates was taken as minimum bactericidal concentration. The determinations were performed in triplicate and the means of three independent experiments were calculated.

Growth experiments were conducted without or with 1% of CNCs in a solution containing 1 x 10^6 cells of *E. coli* ATCC 25922, incubated for 24h. After incubation the microbial suspensions were plated on LB agar plates. Counts were carried out at 48h and 96h. Results are reported in Table S1. After 48h no colony were detected in the plates containing the microbial suspension incubated with the CNCs, but in the same plates, after 96h, the initial bacterial number was observed.

Counts	**T0**	**48h**	**96h**
No CNCs	1E+06	9.0E+08	
1% CNCs	1E+06	No colony	1.2E+06

Table S1: Bacterial growth with or without CNCs

Cell culture:

HT-29 (ATCC® HTB-38™) colon cancer cell line was grown in DMEM medium supplemented with heat-inactivated 10% FBS, 2 mM L-glutamine, and maintained at 37°C in a humidified 5% CO_2 incubator. During routine culture we also used 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were grown in 75 cm^2 flasks and subcultured every time they reach confluence. All the reagents for cell culture were supplied by Lonza (Lonza Group, Basel, Switzerland).

In order to perform bacterial adhesion experiments, the cells were incubated with 0.05% trypsin-EDTA for 5 min and then fresh warm medium supplemented with 10% FBS and 2 mM L-glutamine but containing no antibiotics was added. The cell suspension was homogenized by repeated up-and-down pipetting and centrifuged at 1200 rpm for 10 min. The pellet was resuspended in the same medium and the cells were seeded at a density of 2.5×10^4 cells/well into a 96-well plate and incubated for 24 h in a cell culture incubator.

Bacterial adhesion:

Bacterial adhesion assay was performed to evaluate the effect of cellulose nanoparticles (CNCs) on adherence of *E. coli* ATCC 25922 on a monolayer of human epithelial intestinal HT29 cells. The monolayer of HT29 cells in DMEM medium was prepared in a 96 multi-well plate as described above and left in incubation for 24h at 37°C. Then the microbial suspension containing 1 x 106 cells of *E. coli* pre-incubated with CNCs at two different times of incubation 2h and 24h was added on the monolayer in PBS. A fresh culture of *E. coli* on LB broth was prepared and when the culture reached the O.D. of 0.7 at 600 nm, cells (0.1 mL of the culture) were collected by centrifugation at 12000 rpm for 2 min and pre-incubated in 1 mL of nanoparticles suspension at the concentration of 0.1%. Then the suspension of *E. coli* pre-incubated with CNCs was centrifuged at 12000 rpm for 2 min, to remove CNCs not adhering to bacterial cells, and re-suspended in 1 mL of PBS. The supernatant of the prepared 96 multi-well plate containing the monolayer was removed and 100 µl of *E. coli* pre-incubated with CNCs suspension was added. The 96 multi-well plate containing monolayer bacteria and CNCs were incubated under a 5% CO_2 atmosphere for 3h at 37°C. After incubation the supernatant containing bacteria in suspension was removed and a washing procedure with PBS was performed. Then 100 µL of 1% TritonX100 solution with incubation for 10 min at room temperature was used for the detachment of the attached cells to HT29 from the surface. At this point we determined the number of bacteria by counts on plates. A serial dilution of microbial suspension was performed and plated on TSA agar medium; after over-night incubation at 37° C the CFU/ml of bacteria were determined. Planktonic and adhered cells were quantified using the CFU/ml counts and expressed in percentage.

A1	1,13E+07	**B1**	1,26E+07	**C1**	4,27E+07	**Inoculum**
A2	2,70E+05	**B2**	5,70E+05	**C2**	3,60E+05	***E. coli* Control**
A3	3,85E+03	**B3**	7,05E+03	**C3**	4,00E+02	***E. coli* + CNCs 2h**
A4	5,60E+03	**B4**	5,40E+03	**C4**	3,60E+03	***E. coli* + CNCs 24h**

Table S2: Inhibitory effect of nanoparticles on adherence of *E. coli* to monolayer of HT29 cells (figure 3 main text).