Electronic Supplementary Information (ESI)
for

A new pathway towards polymer modified cellulose nanocrystals *via* a "grafting onto" process for drug delivery

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Experimental Section

Materials

Cellulose nanocrystals (CNCs) hydrolyzed from wood pulp with an average charge density of 0.26 mmol/g was provided by CelluForce Inc. *p*-Toluenesulfonyl chloride (TsCl, 98%) and *N, N, N′, N″, N‴*-pentamethyldiethylenetriamine (PMDETA, 98%) were purchased from J&K Chemical and used as received. Stannous octoate [$Sn(Oct)_2$, 95%, Sigma-Aldrich] and triethylamine (TEA, Sinopharm Chemical Reagent) were distilled under vacuum before use. All the other reagents and solvents were purchased from Sinopharm Chemical Reagent and used as received unless otherwise stated. 2-Ethoxy-2-oxo-1, 3, 2-dioxaphospholane (EOP) was synthesized according to a previously described method and distilled under vacuum.[1,2] Doxorubicin hydrochloride (DOX·HCl, 99%, Beijing Zhongshuo Pharmaceutical Technology Development) and sodium azide (NaN_3, Sinopharm Chemical Reagent) were used without any further purification. Propargyl alcohol (98%, Alfa Aesar), triethylamine (TEA, Sinopharm Chemical Reagent) were distilled under reduced pressure before use. Cuprous bromide (CuBr, 95%, Sinopharm Chemical Reagent) was successively washed three times with glacial acetic acid and acetone, followed by drying for 12 h under vacuum at room temperature. Tetrahydrofuran (THF) was dried over potassium hydroxide for at least two days and then refluxed over sodium wire with benzophenone as the indicator until the colour turned to purple. *N, N*-dimethylformamide (DMF) was dried over anhydrous $MgSO_4$ and distilled before use. Copper salt absorbent (CupriSorb^{TM}) was purchased from Seachem Laboratories and used as received. Milli-Q water (18.2 MΩ cm^{-1}) was generated using a water purification system (Simplicity UV, Millipore). All the cell culture related reagents were purchased from Invitrogen.

Synthesis of CNC-N_3

Cellulose nanocrystals (1.0 g) was refluxed with 100 mL of HCl (0.025 mol L^{-1}) at 80 °C for 20 h and followed by quenching in an ice bath. The desulfonated cellulose nanocrystals was collected by dialysis (MWCO = 20 000) against Milli-Q Water until it was neutralized. The solution was then concentrated and freeze-dried to get partially desulfonated cellulose nanocrystals (abbreviated as CNC, 0.909 g, yield: 90.9 %).

The mixture of CNC (0.54 g) and TsCl (1.2 g) were added into a 50 mL round bottom flask with 10 mL of pyridine. The reaction was performed in an ice bath over 30 min. After stirring at 25 °C for 40 h, the resulting solution was added into 100 ml of anhydrous ethanol and centrifuged. The solid crude product was then washed by anhydrous ethanol for five times and dried under vacuum at 35 °C for 24 h to yield chloride-modified CNC (abbreviated as CNC-Cl, 0.45 g)

Afterwards, CNC-Cl (0.248 g) and NaN_3 (0.25 mg) were added into a 50 mL flask containing 10 mL anhydrous DMF. The reaction was performed at 80 °C for 48 h. The resultant solution was purified by dialysis (MWCO = 20 000) against Mill-Q Water for 48 h. After freeze-drying, the resultant azide-modified CNC was collected as a white powder (abbreviated as CNC-N_3, 0.209 g).

Synthesis of propargyl-PEEP

The propargyl-terminated PEEP (propargyl-PEEP) was synthesized *via* the ROP reaction of EOP monomer according to the previously reported method.[2] Briefly, a 50 mL dry flask containing 15 mL of anhydrous THF was charged with $Sn(Oct)_2$ (0.325 g, 0.8 mmol), propargyl alcohol (0.09 mg, 1.6 mmol) and EOP (5.09 g, 33.5 mmol), which was then

degassed through three exhausting-refilling nitrogen cycles. The mixture was then stirred at 35 °C for 3 h under a nitrogen atmosphere. The resultant solution was concentrated and precipitated twice in cold diethyl ether, and the precipitate was dried under vacuum at 25 °C to obtain the viscous product (abbreviated as propargyl-PEEP, 4.73 g, yield: 92 %).

Synthesis of CNC-*g*-PEEP

The surface PEEP-grafted CNC was prepared *via* CuAAC "click" chemistry between CNC-N_3 and propargyl-PEEP. The detailed synthesis route is described as follows. All magnetic stirring bars and glassware used in the experiments were dried at 120 °C for 24 h and cooled under vacuum to eliminate the moisture before use. CNC-N_3 (0.107 g) and propargyl-PEEP (0.107 g) were dissolved in a nitrogen-purged flask containing 25 mL of anhydrous DMF. Then, CuBr (0.064 g, 0.44 mmol) and PMDETA (90 μL, 0.45 mmol) were added sequentially into the flask. After being degassed through three exhausting-refilling nitrogen cycles, the mixture was stirred under a nitrogen atmosphere at 60 °C for 24 h. The mixture was then exposed to air to terminate the reaction and concentrated under reduced pressure, followed by dialysis (MWCO = 20 000) against a mixed solvent of ethanol and Mill-Q Water with the volume ration from 3/1 to 0/1 (added some CupriSorb™ absorbents at 0/1 ratio). Finally, the solution was freeze-dried to obtain the PEEP-modified CNC (abbreviated as CNC-*g*-PEEP, 0.0758 g).

The mole number of grafted PEEP chains and the grafting efficiency (GE) of PEEP could be calculated by the followed equations:

$$n_{PEEP} = n_{azide} = \frac{m_{CNC\text{-}N_3} \times (\text{N content})}{\text{molecular weight of azide group}}$$

$$GE_{PEEP}\ (\%) = \frac{\text{mole number of grafted PEEP}}{\text{mole number of PEEP in feed}} \times 100$$

Structural Characterizations

^{1}H NMR spectra were recorded on a 400 MHz NMR spectrometer (INVOA-400, Varian) at 25 °C with $CDCl_3$ as solvent and tetramethylsilane (TMS) as the internal standard. The molecular weights ($\overline{M}_{n,NMR}$) of the propargyl-PEEP$_n$ were calculated according to the ^{1}H NMR analysis by the following equation:

$$\overline{M}_{n,NMR} = n \times 152.1 + 56.1; \qquad n = \frac{2A_d}{3A_b}$$

In this equation, 152.1 is the molecular weight of EOP monomer, 56.1 is the molecular weight of the terminal propargyl group and H atom. A_d and A_b are the integral value of the peaks at δ 1.34 (PEEP) and δ 4.68 ppm (HC≡C-C$\boldsymbol{H}_2$-), respectively.

Fourier transform infrared spectroscopy (FT-IR) spectra were determined with a Nicolet 6700 Fourier transform infrared spectrometer using the KBr disk method.

The number-averaged molecular weights ($\overline{M}_{n,GPC}$) and molecular weight distributions (PDIs) of the propargyl-PEEP$_n$ were measured on a Waters 1515 GPC instrument with a Waters 1515 isocratic HPLC pump, a Waters 2414 refractive index detector, a Waters 717 plus autosampler and a set of MZ-Gel SD plus columns (300 × 8.0 mm, 500 Å, 103 Å and 104 Å). GPC measurements were carried out at 35 °C using DMF with 0.05 mol L^{-1} LiBr as the eluent and a flow rate of 0.8 mL min^{-1}. The calibration was carried out with a series of narrowly-distributed polystyrene standards.

Elementary Analysis

Elementary analysis was carried out on an instrument (Vario MICRO Cube, Elementar) to determine the atomic composition (carbon, hydrogen, nitrogen, and sulfur content) for CNCs and samples from each step of azide modification.

Zeta Potential

Zeta potential measurements were performed on an instrument (Zetasizer nano ZS, Malvern) equipped with a dip cell. The concentration of the samples was maintained at 1 mg mL^{-1}.

Self-assembly Behavior

The morphologies of nanocrystals were observed on a TEM instrument (HT7700, Hitachi) at 120 kV. The solution with a concentration of 0.1 mg mL^{-1} was prepared by a direct dispersion method. Briefly, 1 mg of sample was directly dispersed in 10 mL of Milli-Q water and stirred for 48 h before use. Subsequently, 12 μL of the solution was dripped onto the carbon-coated copper grid, and the solvent was evaporated at room temperature before measurement. The morphology was then imaged in a normal TEM at room temperature.

***In Vitro* Drug Loading and Release**

The *in vitro* drug loading and release behavior of the CNC-*g*-PEEP were studied as following. Typically, DOX·HCl (10 mg) with 3 eqv. TEA and CNC-*g*-$PEEP_{29}$ (20 mg) were dissolved in 5 mL of DMF. 10 mL Mill-Q water was added dropwise to the mixture under stirring, followed by dialysis (MWCO 20 000) against Mill-Q Water for 48 h. Subsequently, 5 mL of DOX-loaded solution was placed into a dialysis membrane (MWCO 20 000) and dialyzed against 30 mL of acetate buffer solution (pH 5.0) or PBS buffer solution (pH 7.4) at

37 °C. At the desired time intervals, 5 mL of the released solution was withdrawn for fluorescence measurement (FLS920, Edinburgh) and replaced with 5 mL of fresh buffer solution. The concentration of DOX was determined by fluorescence spectroscopy with excitation at 480 nm and emission wavelength of 590 nm, with a slit width of 5 nm. All the release experiments were carried out in the dark. The DOX loading content (DLC) and DOX loading efficiency (DLE) were calculated according to the following equations:

$$\text{DLC (\%)} = \frac{\text{Weight of DOX loaded in CNC-}g\text{-PEEP}}{\text{Weight of CNC-}g\text{-PEEP}} \times 100$$

$$\text{DLE (\%)} = \frac{\text{Weight of DOX loaded in CNC-}g\text{-PEEP}}{\text{Weight of DOX in feed}} \times 100$$

MTT Assay

The cytotoxicity of CNC, propargyl-PEEP, CNC-*g*-PEEP against HeLa cells and L929 cells was evaluated by MTT assay. The anti-cancer capacity of DOX-loaded CNC-*g*-PEEP was also studied by MTT assay using free DOX as a control. The cells were obtained from American Type Culture Collection (ATCC) and cultured in 10 % heat-inactivated fetal bovine serum (FBS), 1% penicillin and streptomycin contained culture medium at 37 °C under a 5% CO_2 atmosphere. The cells were then seeded in a 96-well plate at a density of about 50 000 cells per well for 24 h. The sample solutions with series of concentration were added to the wells and incubated for another 48 h. After that, 25 μL of the MTT stock solution (5 mg mL^{-1} in PBS) was added into each well. After incubation for additional 4 h, 150 μL of DMSO was added to dissolve the resulted purple formazan. The optical density (OD) was measured on a microplate reader (Bio-Rad 680) at 490 nm. The absorbance values were normalized to the wells where cells were not treated with samples and the data are

presented as the average values with standard deviations.

Cellular Uptake

The cellular uptake of DOX-loaded CNC-*g*-PEEP nanocrystals (DOX content: 2 mg L^{-1}) by HeLa cells was investigated by the live cell imaging system (Cell'R, Olympus). HeLa cells were cultured in the same medium with MTT assay at 37 °C under a 5% CO_2 atmosphere. The culture medium was removed after 24 h incubation. Cells were washed with PBS buffer solution (pH 7.4) and stained with Hoechst 33342 (10 mg L^{-1}) for 30 min. During observation, the cells were incubated with RPMI-1640 culture medium and samples at 37 °C under a 5% CO_2 atmosphere. Images were captured with the excitation wavelength of 340 nm (blue) and 480 nm (red) per 30 min. Furthermore, the cells treated with free DOX (2 mg L^{-1}) were used as a control.

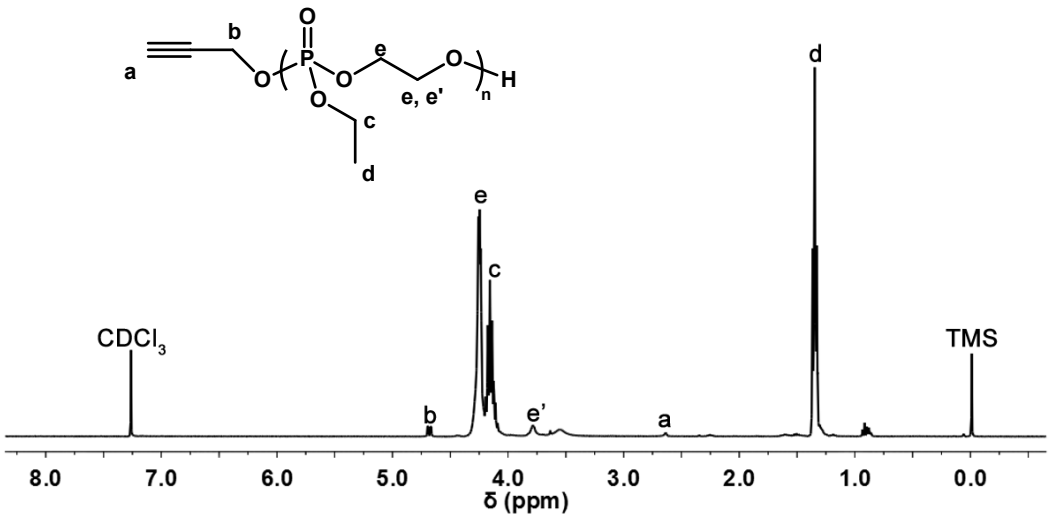

Fig. S1 ^{1}H NMR spectrum of propargyl-$PEEP_{29}$ in $CDCl_3$.

Table S1 Characterization data of the number-average molecular weights and molecular weight distributions (PDIs) of the propargyl-PEEP homopolymers.

Samples	$\overline{M}_{n,thero.}$ [a)] (g mol^{-1})	$\overline{M}_{n,NMR}$ [b)] (g mol^{-1})	$\overline{M}_{n,GPC}$ [c)] (g mol^{-1})	PDI [c)]
propargyl-$PEEP_{29}$	3250	4460	7790	1.13
propargyl-$PEEP_{40}$	5680	6140	11130	1.23

[a)] Theoretical molecular weight.

[b)] Calculated on the basis of ^{1}H NMR measurements in $CDCl_3$.

[c)] Determined by GPC in DMF with polystyrene standards as the calibrations.

Table S2 The atomic compositions of various samples determined by elementary analyses.

Sample	N [%]	C [%]	H [%]	S [%]
Cellulose Nanocrystals	0.00	40.46	6.34	0.64
CNC	0.00	40.57	6.56	0.47
CNC-Cl	0.00	41.52	6.42	0.46
CNC-N_3	0.77	40.56	6.36	0.34

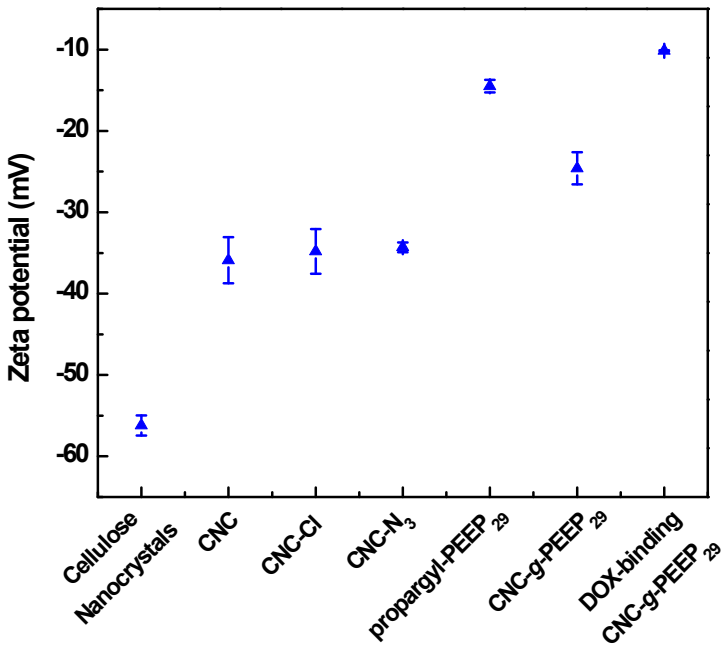

Fig. S2 Zeta-potential (0.1 mg mL^{-1}) of cellulose nanocrystals, CNC, CNC-Cl, CNC-N_3, propargyl-$PEEP_{29}$, CNC-*g*-$PEEP_{29}$ and DOX-binding CNC-*g*-$PEEP_{29}$.