Electronic Supplementary Material (ESI) for Nanoscale.

Supplementary Information

Niclosamide-conjugated polypeptide nanoparticles inhibit Wnt signaling and colon cancer growth.

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Supplementary Methods:

Synthesis of Chimeric Polypeptides. The CP used for conjugation to NIC consists of the sequence SKGPG-$(XGVPG)_{160}$-WPC$(GGC)_7$ (single amino acid codes), where the guest residue X = V: G: A in a 1:7:8 ratio.

Expression and purification of CP. The CP was expressed from a pET-24b expression plasmid transformed into *Escherichia coli* strain BL21(DE3), using a previously published hyperexpression protocol that relies on the leakiness of the T7 promoter[1]. Six 50 mL cultures grown for 16 h were used to inoculate six 1 L flasks of TB dry supplemented with 45 μg/mL kanamycin. Each 1 L flask was incubated for 24 h at 37 °C at 210 rpm, and the cell pellet collected by centrifugation at 3,000 rpm for 10 min at 4 °C. CP was purified using inverse transition cycling (ITC), a non-chromatographic purification method that exploits the temperature-dependent phase transition of CPs, that has been described elsewhere[2]. Briefly, the cell pellet was resuspended in PBS and lysed via sonication on ice for 3 min (10 s on, 40 s off) (Misonix S-4000; Farmingdale, NY). Polyethyleneimine (PEI) 0.7% w/v was added to the lysate to precipitate nucleic acid contaminants. The supernatant was then subjected to repeated rounds of ITC as follows: the solution was heated to 37 °C in the presence of 3 M NaCl to induce coacervation, then centrifuged for 10 min at 14,000 g and 20 °C, and the pellet resuspended in 20 mM TCEP in water, pH 7. This suspension was cooled to 4 °C to induce dissolution of aggregates, and then centrifuged for 10 min at 14,000 and 4 °C to remove any insoluble contaminants. Typically, three rounds of ITC generated a pure product (>95% by SDS-PAGE).

Protein Purity Analysis. Proteins were visualized by Simply Blue Safe Stain (Invitrogen, LC6060)[3]. In brief, proteins were separated on 10% SDS-PAGE mini-gel. After electrophoretic separation, the mini-gel was rinsed with 100ml ultrapure water 3 times for 5 minutes, followed by staining with Simply Blue Safe Stain for at least 1 hour at room temperature with gentle shaking.

Synthesis of CP-NIC Conjugate. NIC (1.032 g, 3.16 mmol) and dry DMF (5 mL) were added to a dry vial. Next, Et_3N (0.4 mL, 2.84 mmol) was added to the suspension, and the mixture was sonicated to produce a red-colored homogeneous solution. DCC (1.95 g, 9.47 mmol), dry DMF (5 mL) and 6-Maleimidohexanoic acid (1.99 g, 9.47 mmol) were added to a dry round-bottomed flask equipped with a magnetic stir bar under an Argon atmosphere. The red DMF suspension of NIC was added dropwise over 2 min to this solution at room temperature, and the vial was rinsed with twice with 1 mL dry DMF that was added to the flask. After 5½ hours, an additional 0.2 mL of Et_3N was added, the reaction mixture was stirred for an additional 15 min, and then filtered to remove a white precipitate that formed during the course of the reaction. The filtrate was poured into 200 mL of 0.1 M NaH_2PO_4 solution at pH 4-5, and the mixture was extracted twice by 75 mL ethyl acetate. The ethyl acetate solutions were combined and washed three times with PBS (pH 4-5), three times with water, twice with 3% sodium bicarbonate (freshly prepared), once with saturated sodium chloride solution, then dried over sodium sulfate and filtered. To the filtrate was added 5 mL of silica gel, and ca. 25 mL heptane, and the mixture was concentrated to dryness on a rotary evaporator. The solids were loaded onto a 120 mL silica gel column packed in 1% ethyl acetate/chloroform, and eluted with a gradient of 1-4 % ethyl acetate/chloroform. The fractions containing the desired material (R_f = 0.2 in 4 % EtOAc/$CHCl_3$) were combined and concentrated to give 1.34 g (82%) of the 6-Maleimidohexanoic ester of NIC (4-chloro-2-((2-chloro-4-

nitrophenyl)carbamoyl)phenyl 6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)hexanoate, compound I in Supplementary Figure 1) as a pale yellow solid.

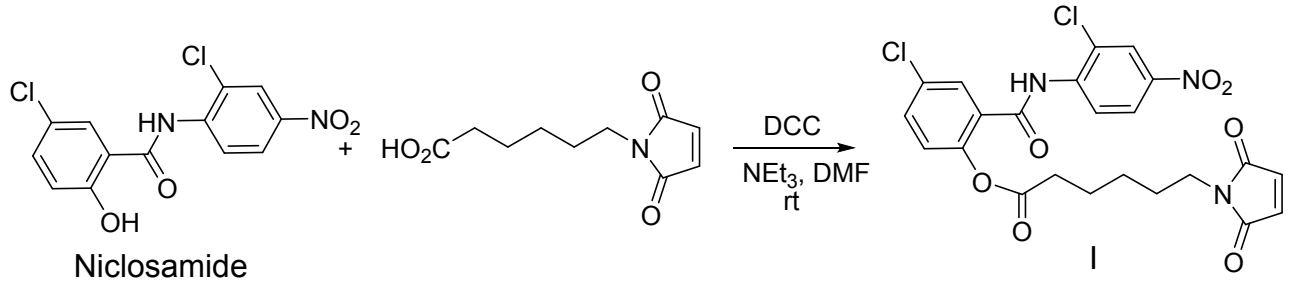
^{1}H NMR (400 MHz, DMSO-d_6) δ = 10.44 (br. s, 1H), 8.36 (d, *J* = 2.54 Hz, 1H), 8.23 (dd, *J* = 2.60, 9.20 Hz, 1H), 8.05 (d, *J* = 9.08 Hz, 1H), 7.79 (d, *J* = 2.54 Hz, 1H), 7.66 (dd, *J* = 2.54, 8.7 Hz, 1H), 7.30 (d, *J* = 8.7 Hz, 1H), 6.96 (br. s, 2H), 3.28 (t, *J* = 7.2 Hz, partial overlap with H2O peak), 2.50 (t, *J* = 7.3 Hz partial overlap with DMSO peak), 1.47 - 1.63 (m, 2H), 1.30 - 1.45 (m, 2H), 1.12 -1.26 (m, 2H). MS (ESI) m/z = 518 (M-1). FTIR (thin film, cm^{-1}) υ = 3369 (br, med), 1770 (med), 1702 (st).

Prior to conjugation with the 6-Maleimidohexanoic ester of NIC (compound I), purified CP was suspended in reaction buffer (0.1 M sodium phosphate, 1 mM Ethylenediaminetetraacetic acid (EDTA), pH 7.0) and reduced with 1 mL of Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) at neutral pH (100 mM, pH 7.0) at ~5x excess to thiol. Excess TCEP was removed from the solution by initiating the phase transition with sodium chloride (2.5 M) and centrifugation at 4,000 rpm at 25 °C for 10 minutes. The CP pellet obtained by centrifugation was re-suspended in ~2 mL of reaction buffer. 6-Maleimidohexanoic ester of NIC (compound I). Purified Nic-ε-maleimidocaproic acid (NIC-EMCA was suspended in ~2 mL of DMF and slowly transferred to the stirring CP solution. 1 mL of pH neutral TCEP (100 mM) was added and the reactants were stirred for 16 hrs at 20 °C in the dark. After reaction, the unreacted Niclosamide 6-Maleimidohexanoic ester precipitate was separated by centrifugation at 13,000 rpm at 10 °C for 10 minutes. The supernatant was further purified by diluting it in 20% acetonitrile in PBS and centrifuging the solution in an Amicon Ultra-15 Centrifugal Filter Units (MWCO: 10KDa, Millipore) at 2,500 rpm at 10 °C. The CP-NIC solution was washed twice with NH_4HCO_3 solution (pH 7.4) and then freeze-dried.

Determination of NIC Conjugation Ratio. The conjugation ratio of NIC to CP was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) of the CP-NIC conjugate and free CP using a Voyager DE-Pro MALDI-MS (Applied Biosystems) instrument equipped with a nitrogen laser (337 nm). The MALDI-TOF-MS samples were prepared in an aqueous 50% acetonitrile solution containing 0.1% trifluoroacetic acid (TFA), using a sinapinic acid matrix. The conjugation ratio was determined by examining the increase in mass of the CP-NIC conjugate relative to unmodified CP.

Temperature Programmed Turbidimetry. The transition temperature (T_t) of each sample was calculated by recording the optical density at 650 nm as a function of temperature (1 °C/min ramp) on a temperature controlled UV–Vis spectrophotometer (Cary 300 Bio; Varian Instruments, Palo Alto, CA). The T_t was defined as the inflection point of the turbidity profile. All samples were analyzed in 90% mouse serum with CP concentrations in the range of 5-50 μM.

Determination of CMC. CP-NIC was characterized by fluorescence spectroscopy using pyrene as a probe of local hydrophobicity, which enables measurement of the critical micelle concentration (CMC) of CP-NIC micelles. The ratio of the first fluorescence emission peak ($I_{370-373}$) and the third peak ($I_{381-384}$) were plotted over a range of CP-NIC concentrations. The sigmoid of best fit was used to calculate the CMC, defined as the inflection point of the curve.



Niclosamide

I

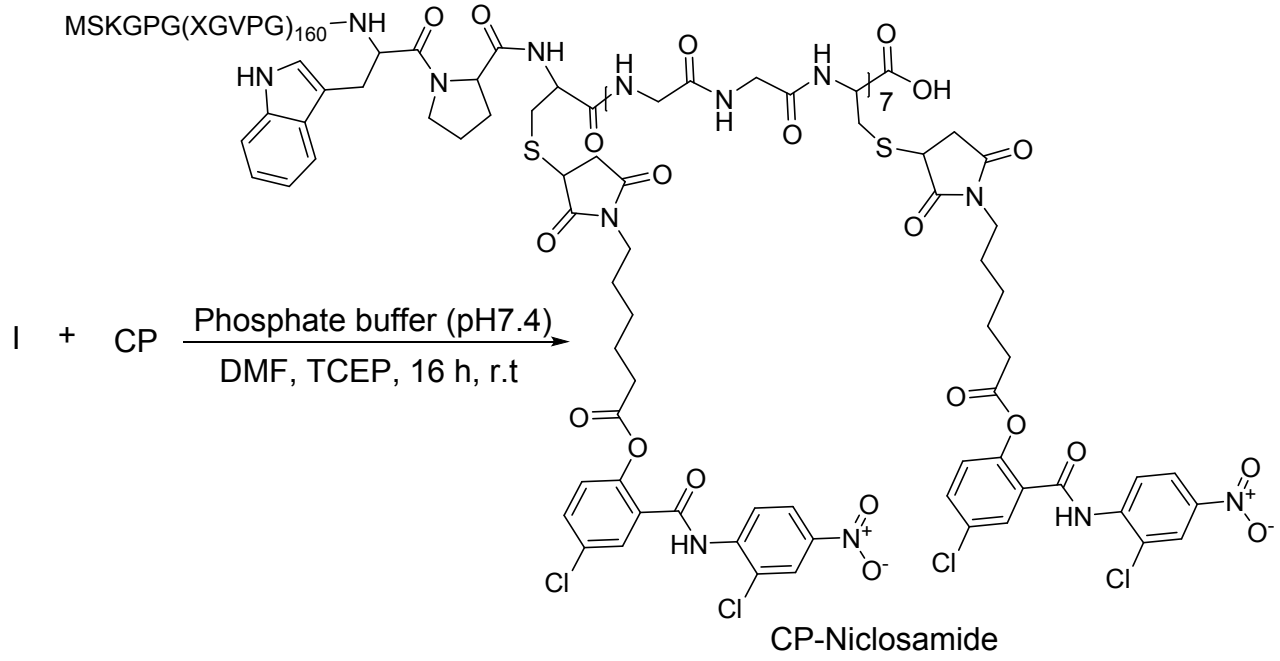


Figure S1. **Synthesis of CP-NIC conjugate**. A terminal maleimide was added to NIC via a substituted hexanoic acid to enable conjugation of NIC to the polypeptide. Treatment of NIC with 6-Maleimidohexanoic acid and N,N'-dicyclohexylcarbodiimide (DCC) produced the 6-Maleimidohexanoic ester derivative of NIC (**I**), which was covalently attached to the Cys residues of the CP.

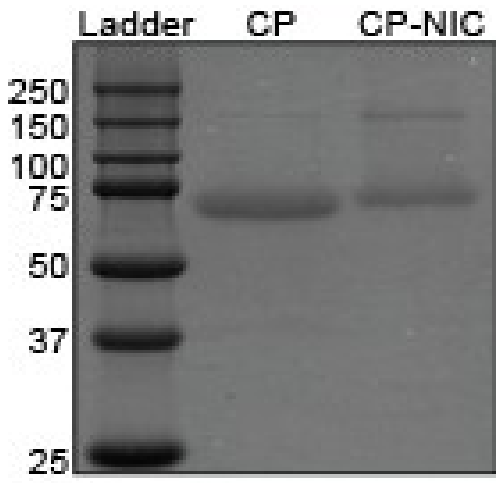


Figure S2. **Determination of the purity of CP and CP-NIC**. SDS-PAGE of CP and CP-NIC conjugate.

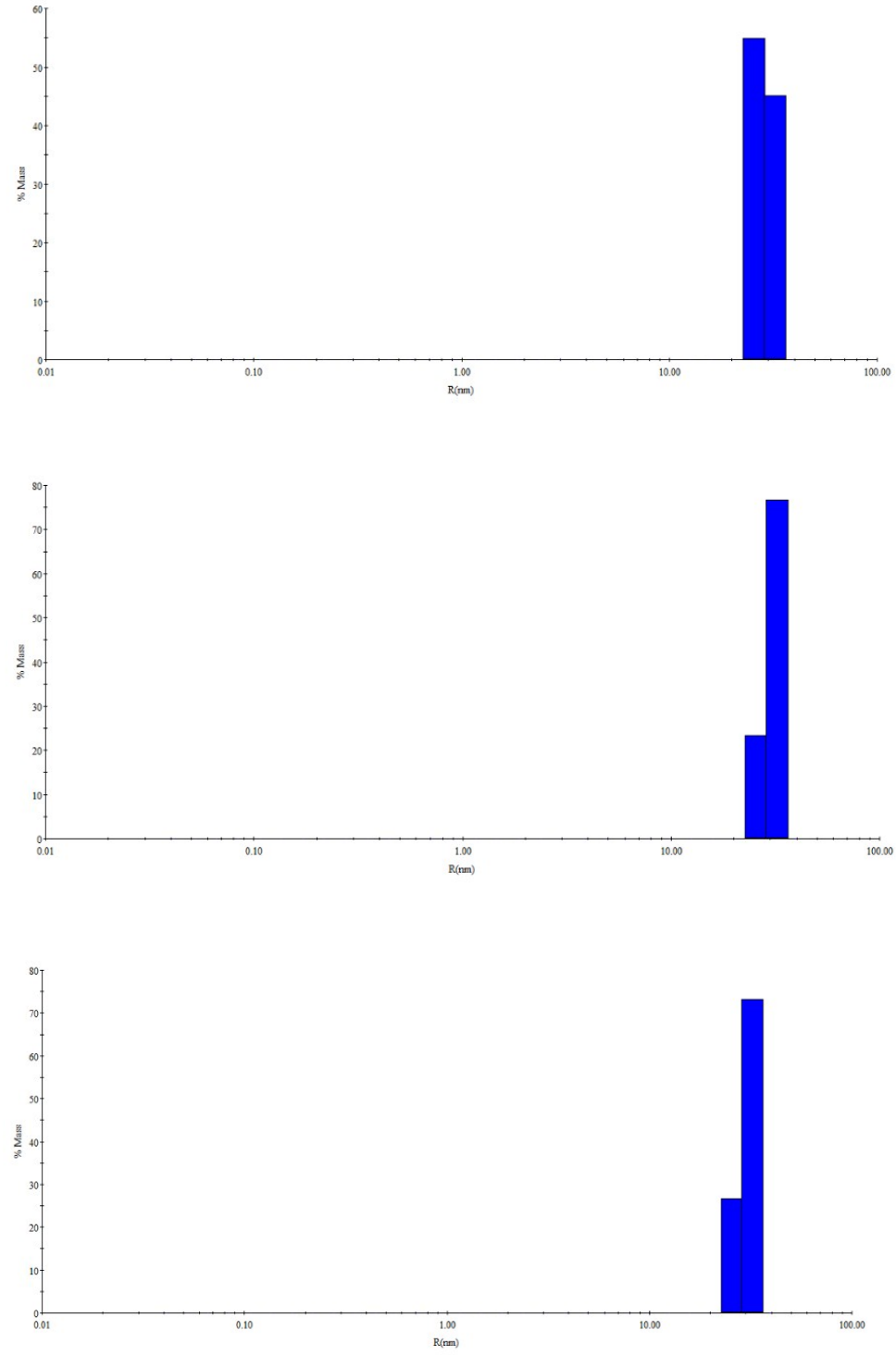
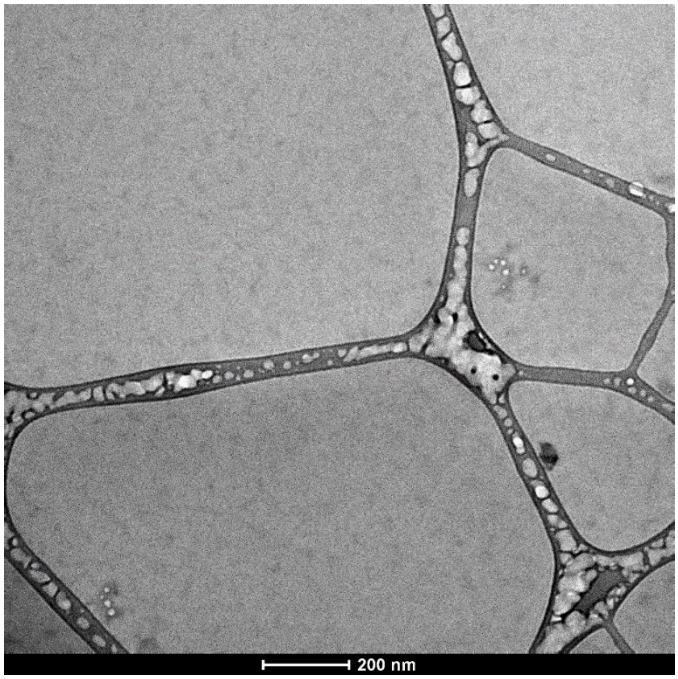


Figure S3. DLS of CP-NIC conjugate. Dynamic light scattering was used to measure particle radius at 25 °C and at 10 µM concentration in PBS after filtration through an AnotopTM syringe filter with 0.22 µm size pores (Whatman; Florham Park, NJ) using a DynaProTM Plate Reader (Wyatt Technology; Santa Barbara, CA). The histograms (n=3) were obtained after regularization fits to determine the hydrodynamic radius as weighted by the percent by mass.

a.



b.

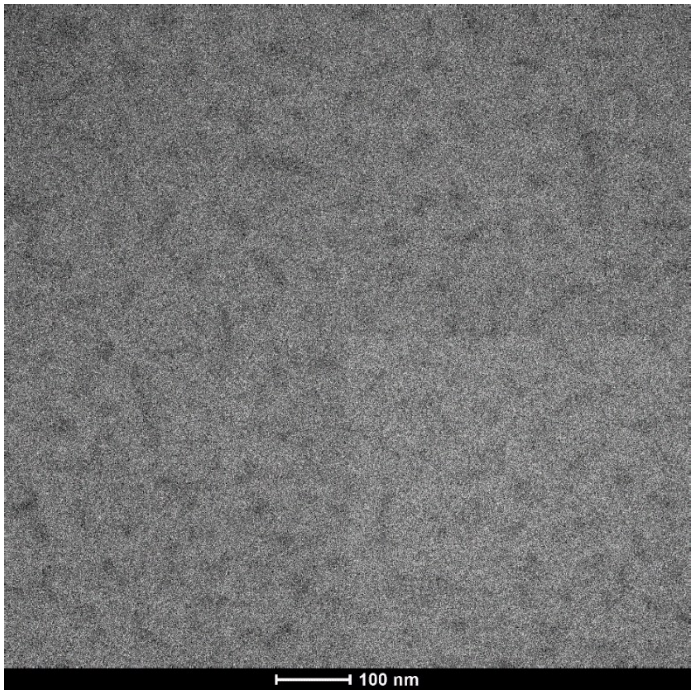


Figure S4. Cryo-TEM micrograph of CP-NIC conjugate.

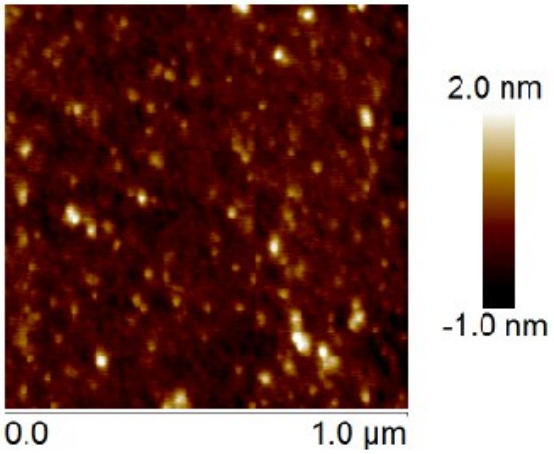


Figure S5. AFM images of CP.

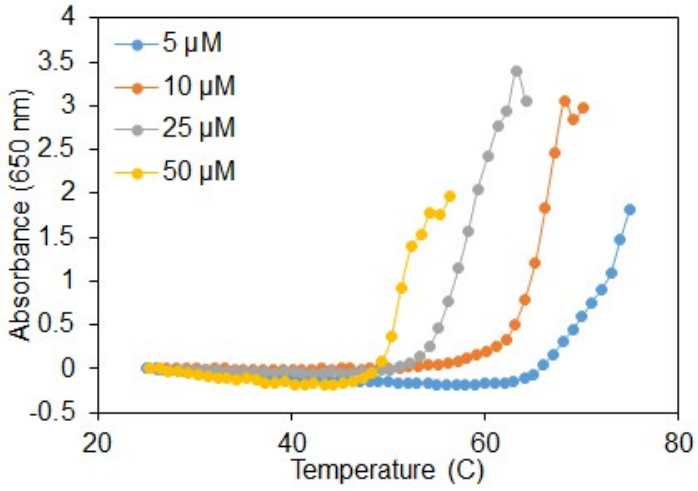


Figure S6. Determination of transition temperature (T_t) of CP at concentration ranging from 5-50 µM in PBS.

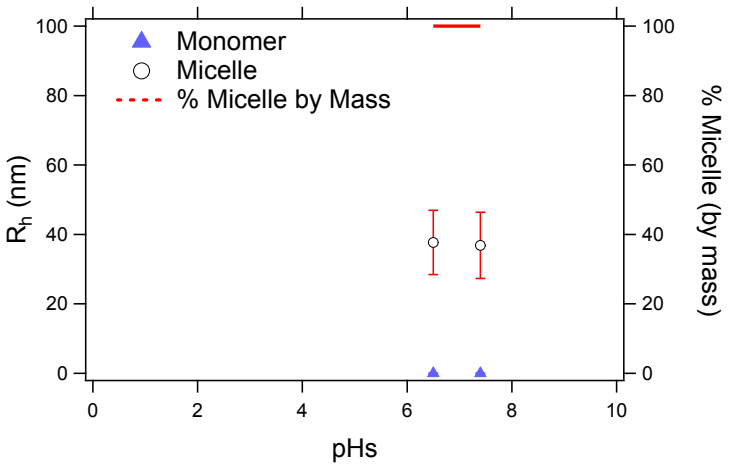


Figure S7. Determination of the hydrodynamic radius of NIC from CP-NIC at pH7.4 and 6.5 and at 25 ^{o}C.

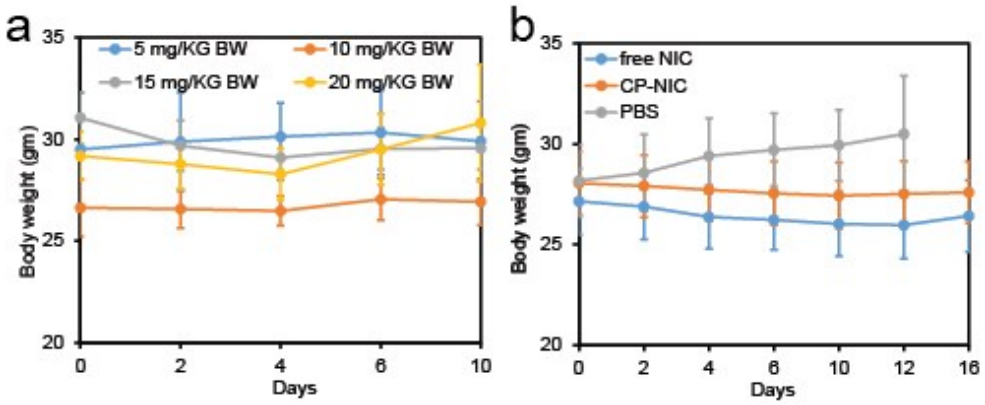


Figure S8. **Change in body weight of mice**. (**a**) Dose escalation in mice bearing subcutaneous HCT116 tumor. Solutions were administered starting at day 0. Mice were treated intravenously q3d for two weeks with CP-NIC at 5-20 mg NIC equiv/kg BW. Points represent the mean ± SD (n=4). **(b**) Body weight of mice (up to 16 days) bearing subcutaneous HCT116 tumor and were treated with CP-NIC, unconjugated NIC, and PBS as mentioned in figure 5 a-b for two weeks with PBS (*n*=8), unconjugated NIC (5 mg/kg BW, *n*=8) or CP–NIC (20 mg NIC equiv/kg BW, *n*=8).

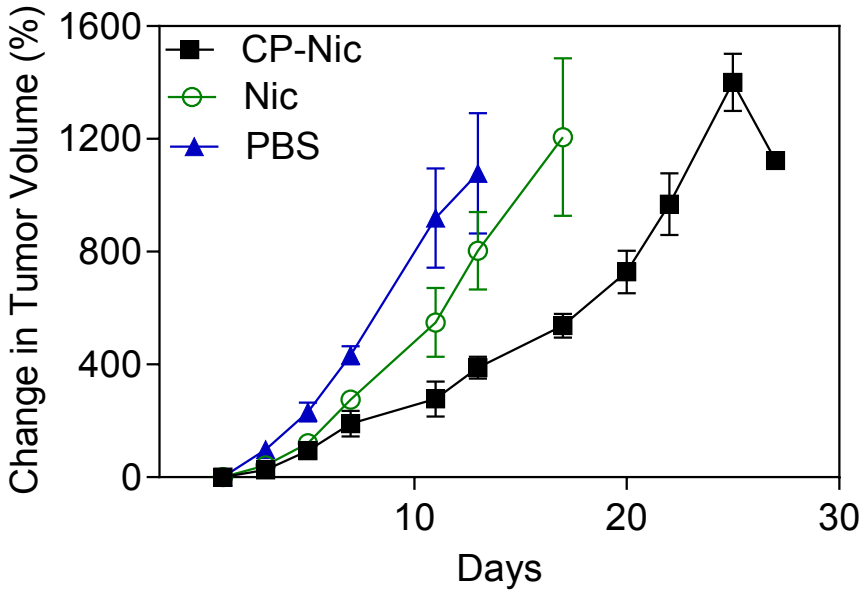


Figure S9. ***In vivo*** **anti-tumor activity of CP–NIC nanoparticles.** % change in the tumor volume up to day 30 (mean ± 95% CI, *n*= 8) with mice bearing subcutaneous HCT116 tumor were treated intravenously every third day for two weeks with PBS (*n*=8), unconjugated NIC (5 mg/kg BW, *n*=8) or CP–NIC (20 mg NIC equiv/kg BW, *n*=8) as mentioned in Figure 5.

Table S1. **Pharmacokinetic parameters of NIC delivered by CP-NIC nanoparticles, and free drug.**

PK parameter	NIC	CP-NIC
C_{max}, mg/mL	6.2 ± 4.23	28.7 ± 32.16
t_{max}, h	0.2 ± 0.17	0.04 ± 0.08
AUC_{last} (area under curve, up to last measured point), h*mg/mL	3.3 ± 1.30	36.9 ± 7.34
AUC_{inf} (extrap. to infinity), h*mg/mL	3.3 ± 1.31	37.7 ± 7.73
% AUC-inf extrapolated ((AUC-last/AUC-inf) *100	0.33 ± 0.45	2.1 ± 1.33
$t_{1/2}$ (half-life of the terminal process), h	1.0 ± 0.22	4.2 ± 1.34
CL (clearance = dose/AUC-inf), L/h (per kg BW)	0.89 ± 0.45	0.07 ± 0.01
MRT_{last} (mean residence in body, up to last measured point), h	0.9 ± 0.44	5.8 ± 0.95
MRT_{inf} (mean residence in body, extrapolated), h	0.9 ± 0.48	6.4 ± 0.93
V_{ss} (=CL x MRT; overall distrib. Volume at steay state), L (per kg BW)	0.7 ± 0.28	0.5 ± 0.12

References:

[1] Meyer DE, Chilkoti A. Purification of recombinant proteins by fusion with thermally-responsive polypeptides. *Nature biotechnology*. 1999;**17**:1112-5.

[2] MacKay JA, Chen M, McDaniel JR, Liu W, Simnick AJ, Chilkoti A. Self-assembling chimeric polypeptide-doxorubicin conjugate nanoparticles that abolish tumours after a single injection. *Nature materials*. 2009;**8**:993-9.

[3] Steinberg TH. Protein gel staining methods: an introduction and overview. *Methods in enzymology*. 2009;**463**:541-63.