Supporting Information

Defined Host-guest Chemistry on NanoCarbon for Sustained Inhibition of Cancer

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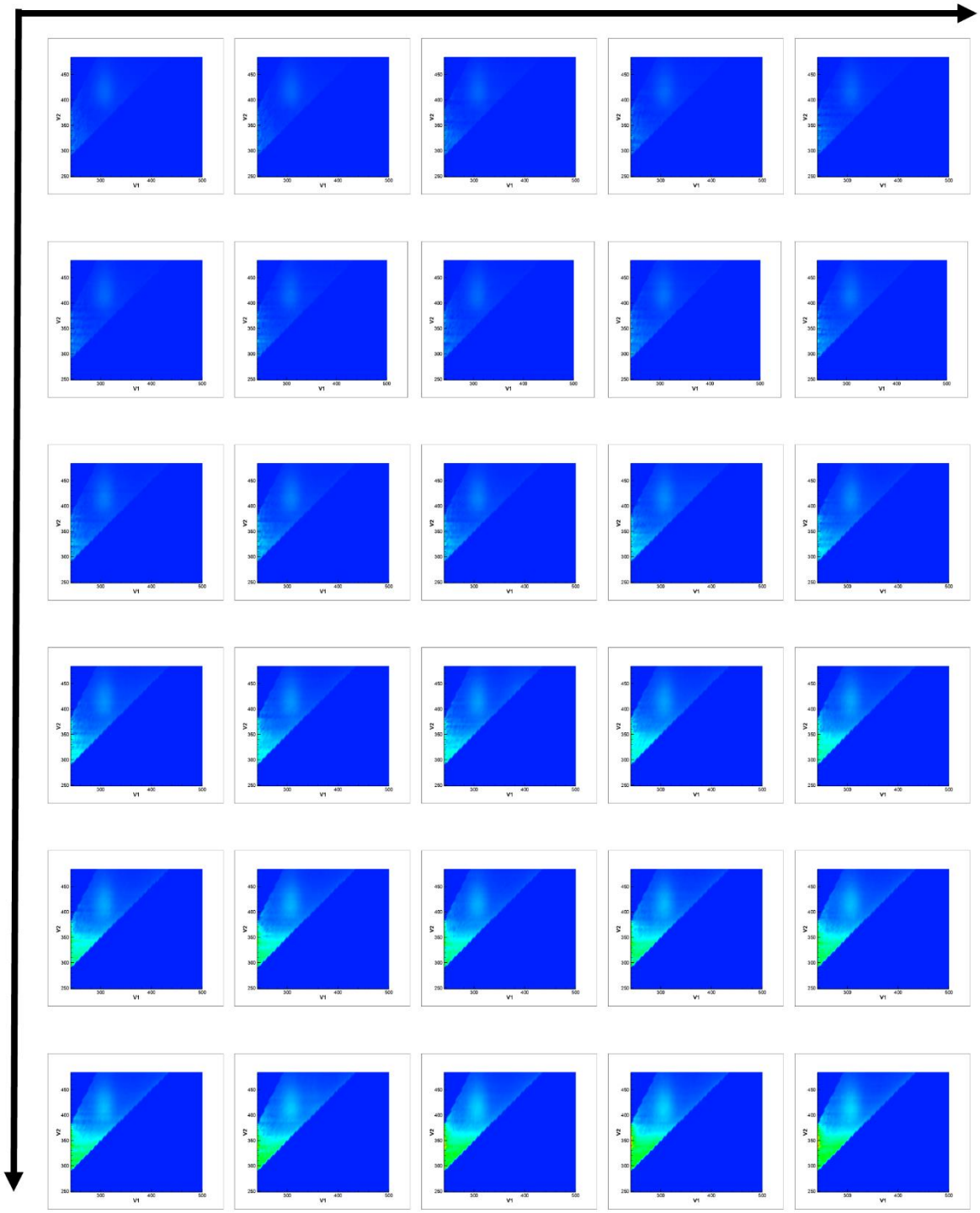
V1
V2

Figure S1. The fluorescence titration of Nic with CB6 observing the scattering pattern. The arrows indicate the increase in concentration of CB6.

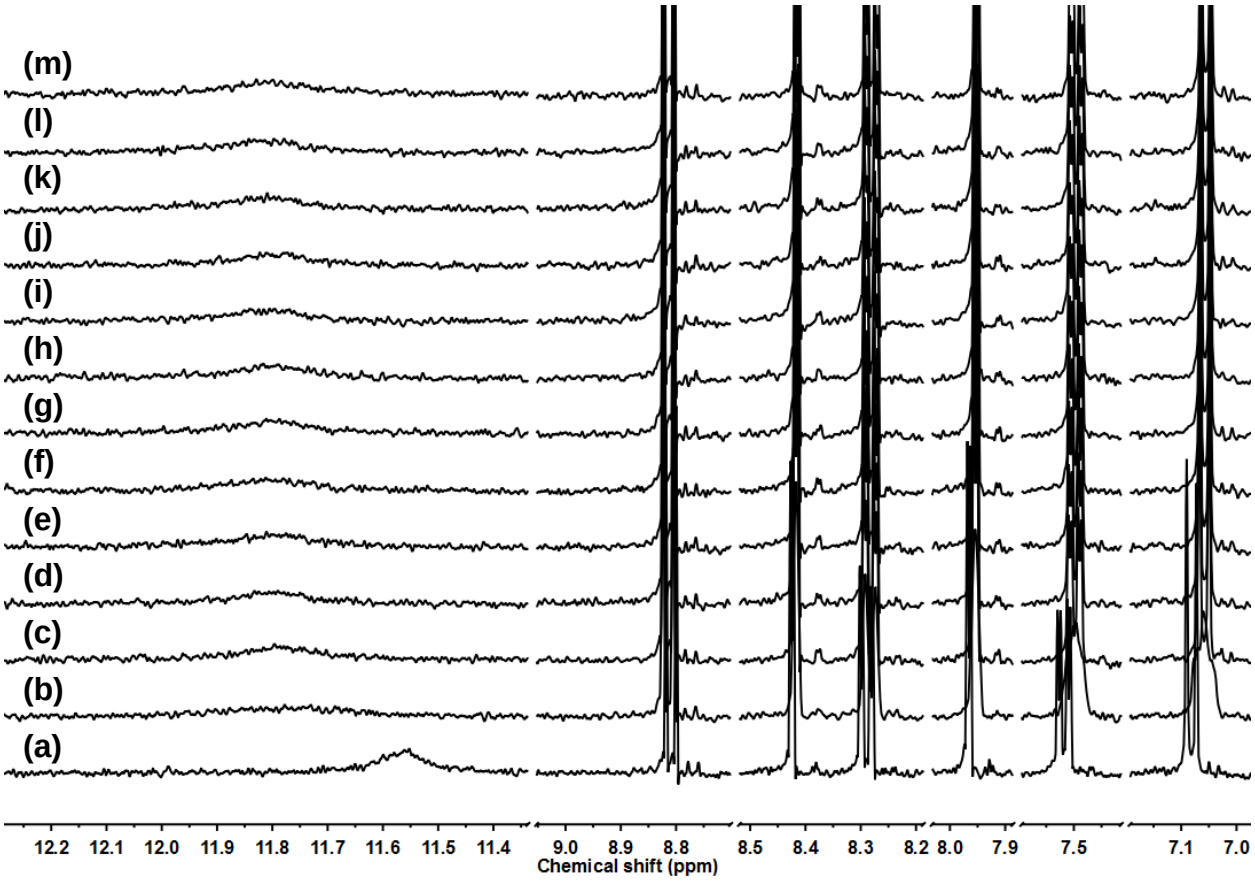

Figure S2. The ^{1}H NMR spectra of niclosamide (2 mM) in the (a) absence and presence of (b) 0.21, (c) 0.42, (d) 0.62, (e) 0.81, (f) 1.00, (g) 1.18, (h) 1.36, (i) 1.54, (j) 1.71, (k) 1.88, (l) 2.04, (m) 2.20 equivalent of CB6.

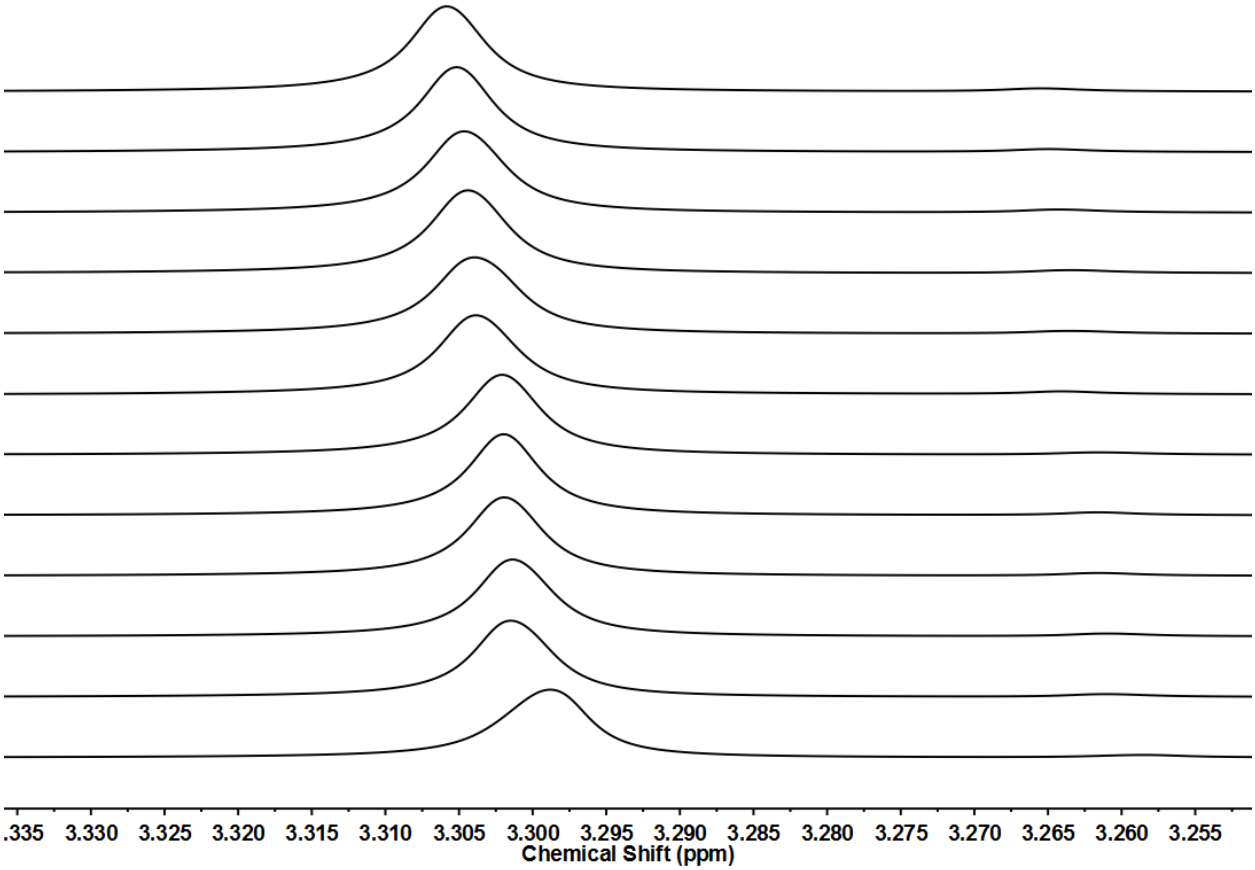

Figure S3. The downfield shift of water peak in ^{1}H NMR titration in the presence of increasing CB6 concentration indicating the release of water molecule upon Nic encapsulation.

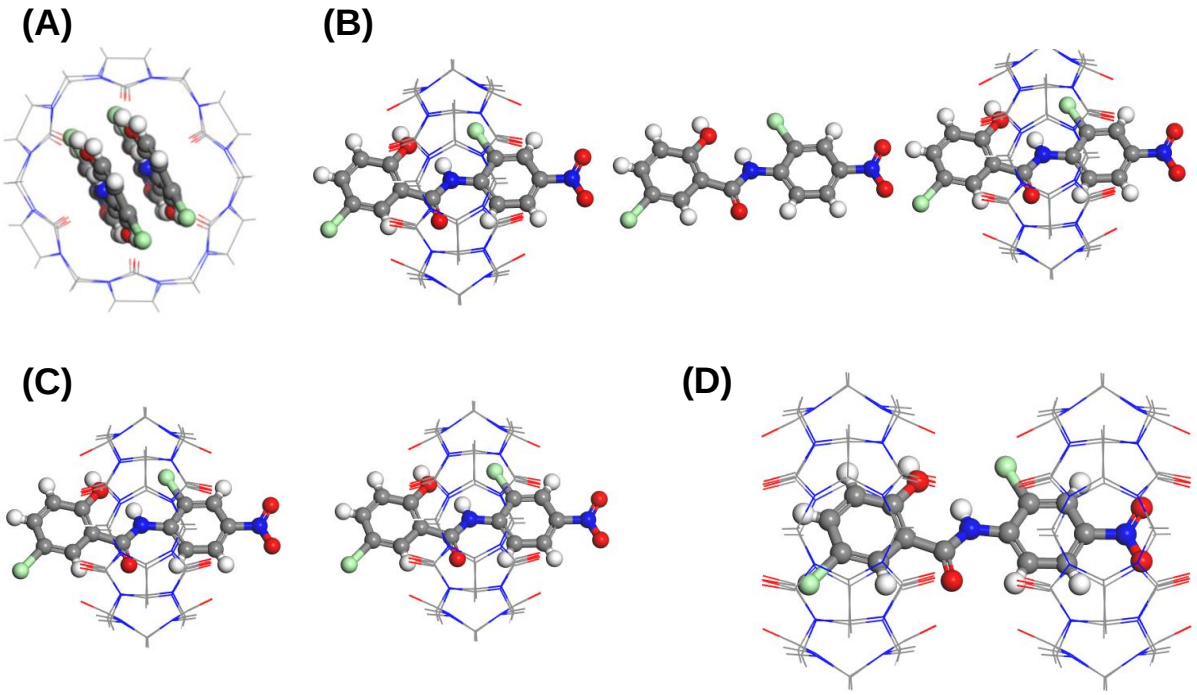

Figure S4. The possible binding scenarios showing the ratio of CB6: Nic (A) 1:2, (B) 2:3, (C) 1:1 (D) 2:1.

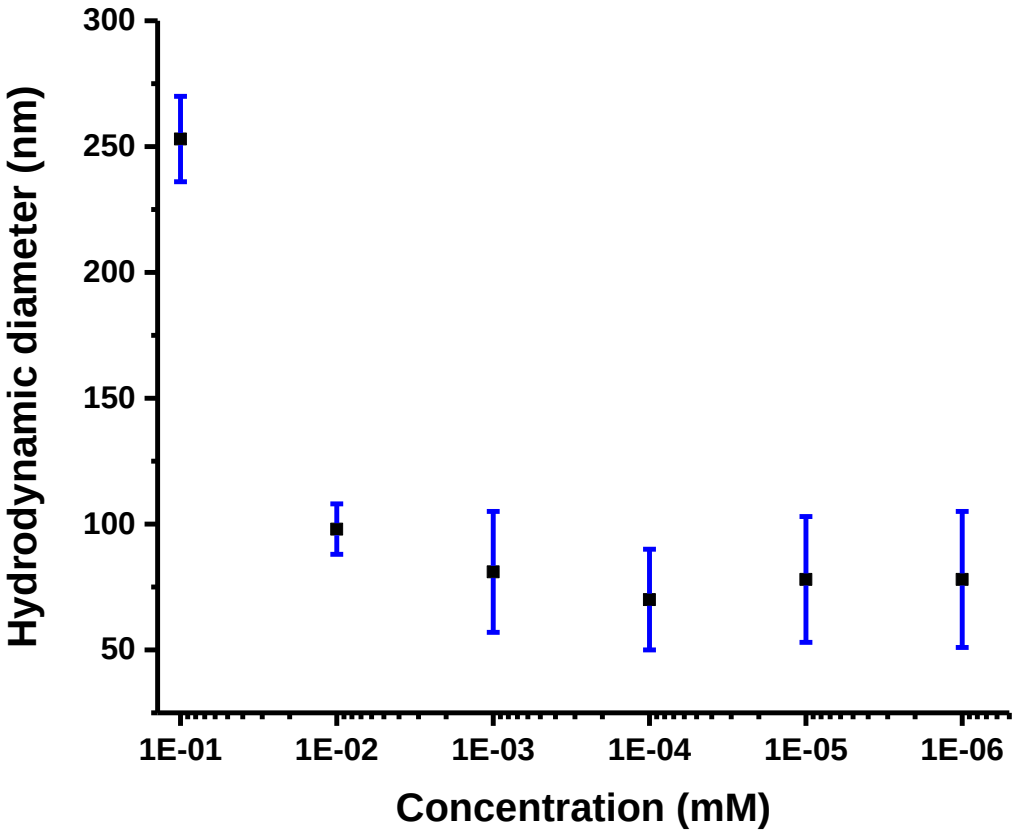

Figure S5. The size optimization of the CB6 CNP post functionalized nanoparticles. The concentration of CB6 is given on the horizontal axis. Further dilution of CB6 down to 10^{-3} mM does not appreciably change the hydrodynamic size of the nanoparticles. Therefore, 10^{-3} mM CB6 was adopted for the preparation of prefunctionalized samples and the rest of the experiments.

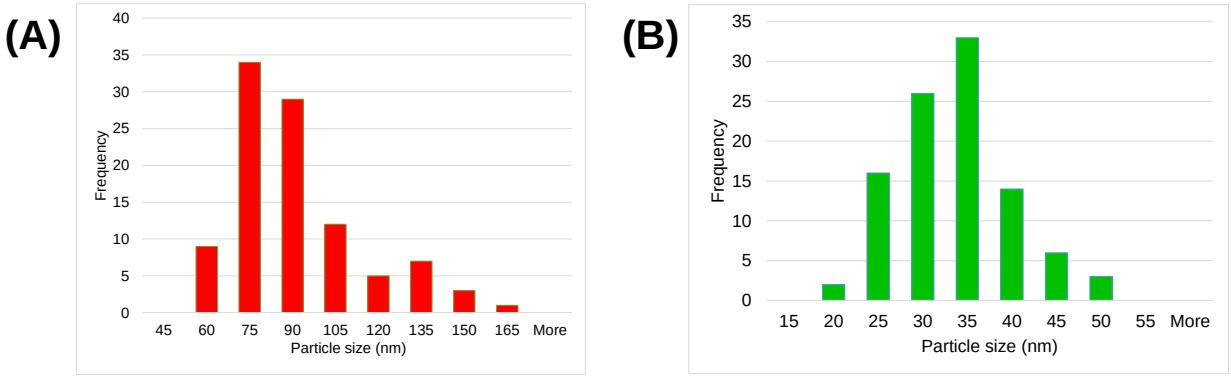

Figure S6. Size distribution of the anhydrous diameter of CB6 CNP (A) post and (B) pre functionalized samples obtained after 100 random measurements from the TEM micrographs.

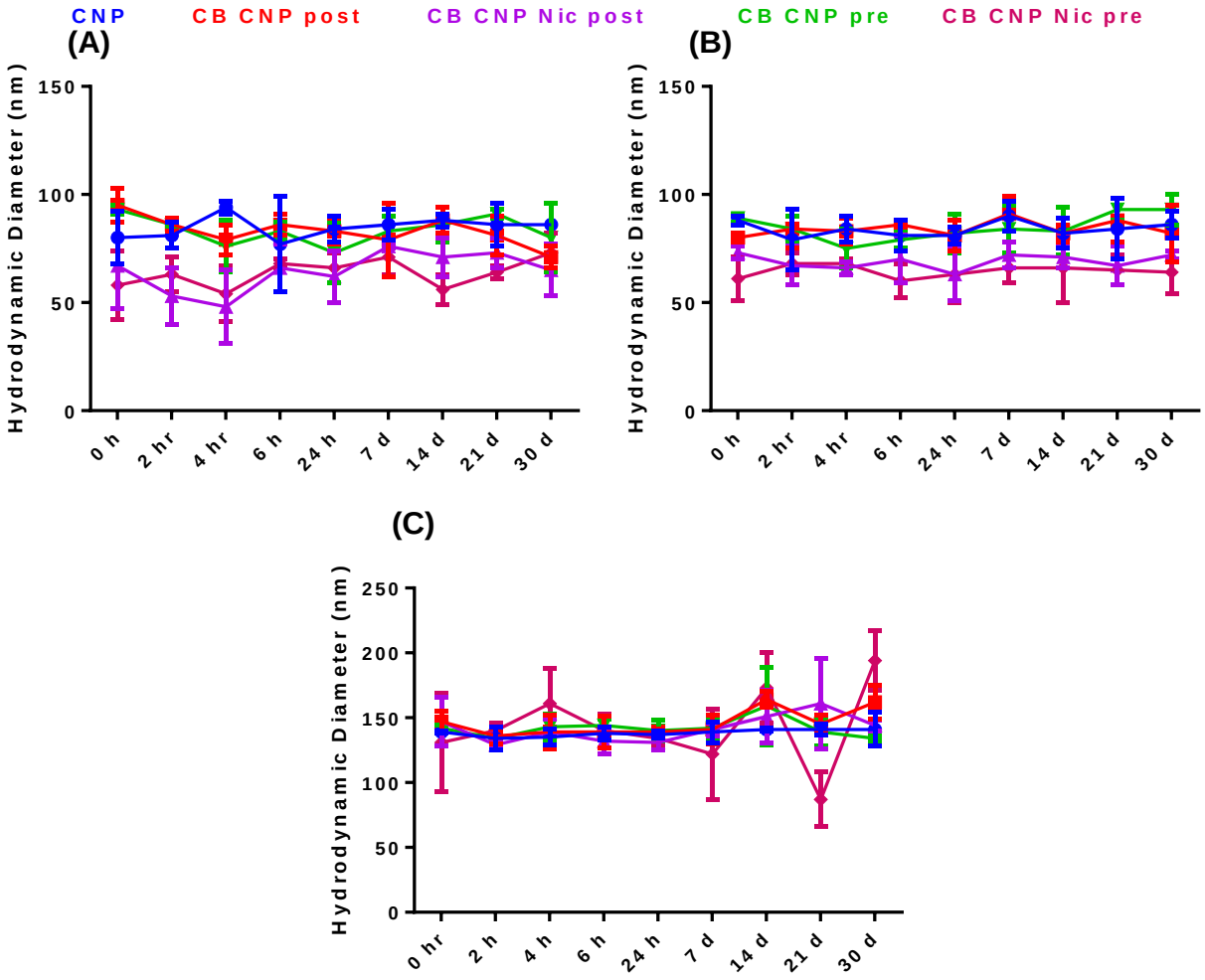

Figure S7. The hydrodynamic size of the nanoparticles were monitored as a measure of shelf life stability over the period of one month in physiologically relevant conditions in aqueous media (A) pH=7.4, (B) pH=4.6 and (C) pH=7.4 with 10% FBS. Note that in (A) and (B) the hydrodynamic size was weighted by number average whereas in (C) the size was intensity weighted. The experiment for measuring the size based on number average was hindered due

to the masking effects of FBS macromolecule creating artifacts in the range of approximately 10 nm.

DRUG DISSOLUTION STUDIES

The drug release profile of CB6 CNP Nic post functionalized sample over 120 h at two different pHs are demonstrated in Figure S7. The phosphate buffer saline (pH=7.4) simulates the normal physiological condition whereas acetate buffer (pH=4.6) was chosen to mimic the intracellular conditions of cancer cells. It is seen that after 5 days, ~ 80% of the drug got released with the remaining cargo to be released in the following days, suggesting sustained release kinetics.

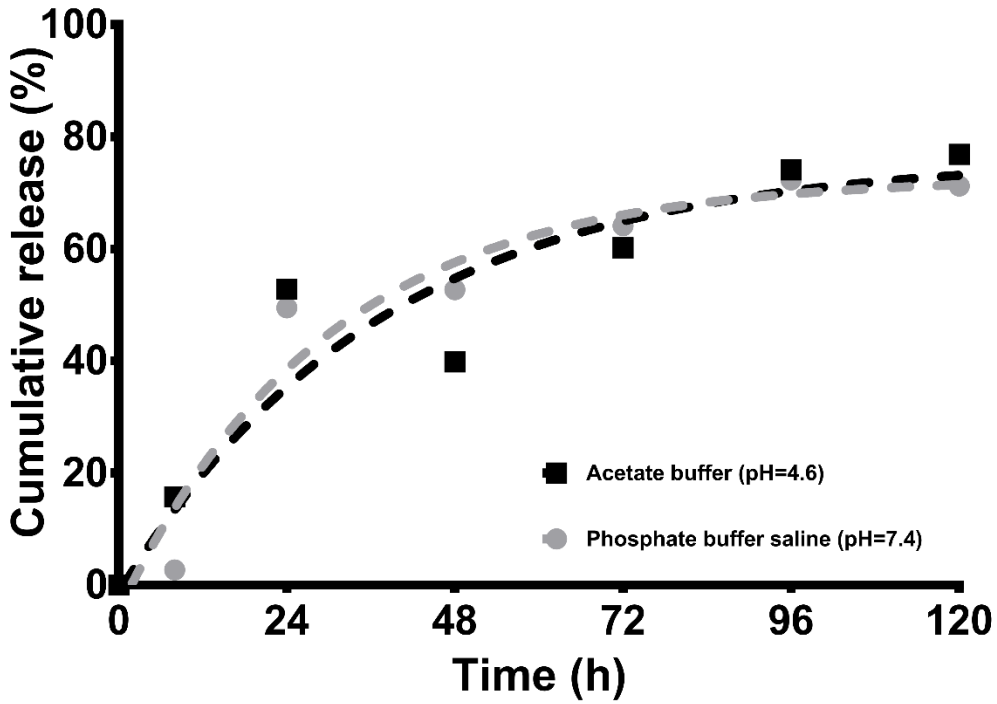

Figure S8. The niclosamide release profile monitored for 5 days in (A) phosphate buffer saline (pH=7.4) representative of normal body condition and (B) acetate buffer (pH=4.6)

mimicking the intracellular conditions of cancer cells. Both of the profiles yield the sustained release with little variation in different pH conditions.

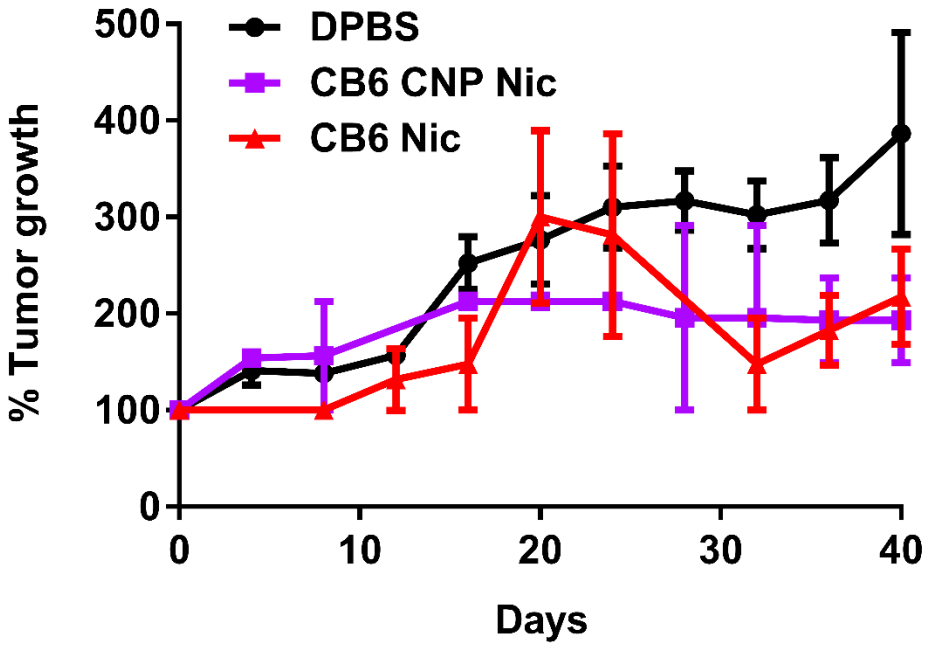

Figure S9. Tumor growth curve monitored over the time frame of the experiment. Note that the growth of the tumor was controlled for the mice treated with either CB6 Nic or CB6 CNP Nic. The trend for tumor growth is more consistent in the case of CB6 CNP Nic compared to CB6 Nic after dose withdrawal on 32nd day.

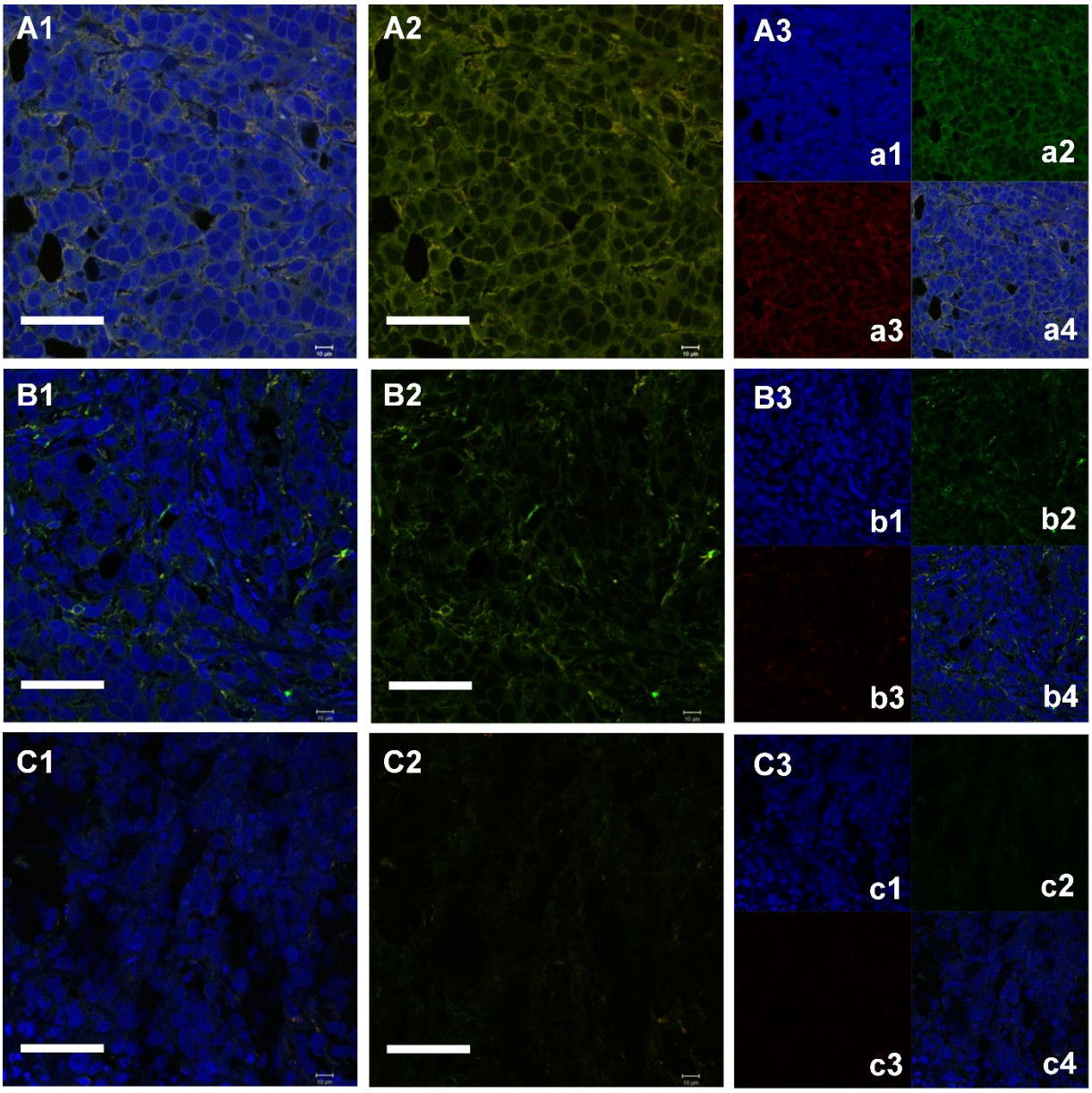

Figure S10. Representative immuno-histochemical analysis of xenograft sections treated with (A1-A3) DPBS, (B1-B3) CB6 Nic and (C1-C3) CB6 CNP Nic observed in animal #7; 29 and 18, respectively. A1, B1 and C1 represent the β-actin stained cells with DAPI stained nucleus; A2, B2, C2 β-actin stained cells without nuclear staining and A3, B3 and C3 for individual

staining of nucleus (a1, b1, c1); β-actin (a2, b2, c2); pSTAT3 (a3, b3, c3) and overlapped images (a4, b4, c4 and d4). It represents decrease in pSTAT3 staining (red) for CB6 Nic and CB6 CNP Nic treated tumor sections.

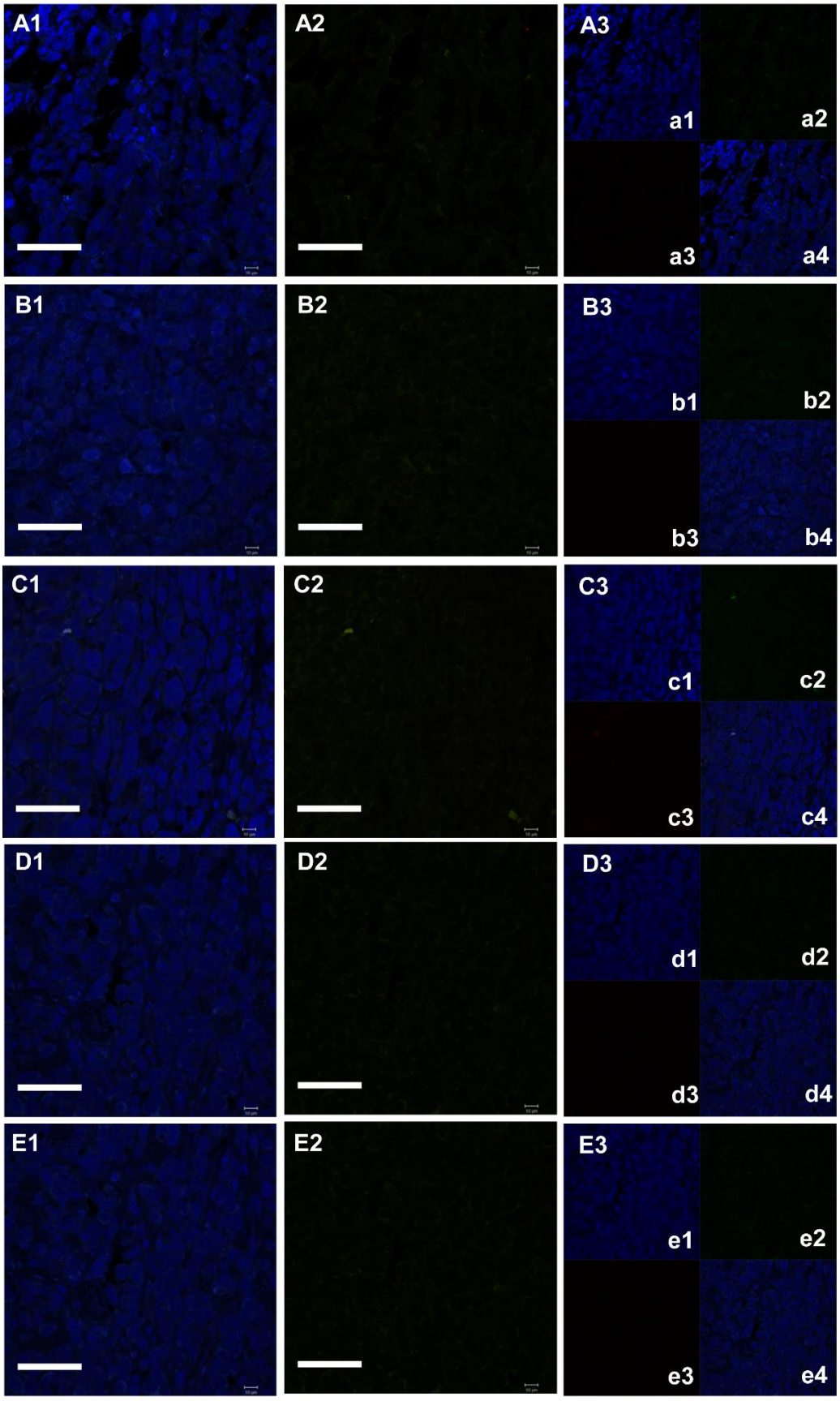
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Figure S11. Representative immuno-histochemical analysis of xenograft sections treated with (A-A3) DPBS, (B1-C3) CB6 Nic and (D1-E3) CB6 CNP Nic observed in animal #7; 5 and 29 and 16 and 18, respectively. These sections were not stained with β-actin and pSTAT3.

To further elucidate the release process of Nic molecule from CB6, we adopted the reaction coordinate of the distance, *r*, between two states, A and B which are shown in Figure S10 A, B. Location A corresponds to the state that amide group of Nic molecule coincides with the mid-point of CB6 molecule while the state B shows the case where Nic is held apart from the CB6 cavity. The energy landscape during the release of guest molecule in the vacuum space is shown in Figure S10 C. Next, the minimized host-guest systems, i.e. 1:1, was immersed in cuboidal box of water molecules. The box of water is prepared prior to starting constrained molecular dynamics (CMD) simulations through a minimization and NPT equilibration at 300K and 1 atm pressure using Nose'-Hoover [*] barostat. In addition, the energetics of complexes in the dispersion during the release process is studied *via* CMD at 300 K. Here, we employed a bond-restraint approach to find the minimum energy pathways (MEP) on the potential energy surface between two sites for Nic molecules extraction that represents stable local energy minima. To explore the migration pathways between two equilibrium states, a penalty potential function is added to the usual ReaxFF energy formulation so as to maintain the distance between the center of mass of Nic molecules and the final configuration in the solution using the **Equation S1**.

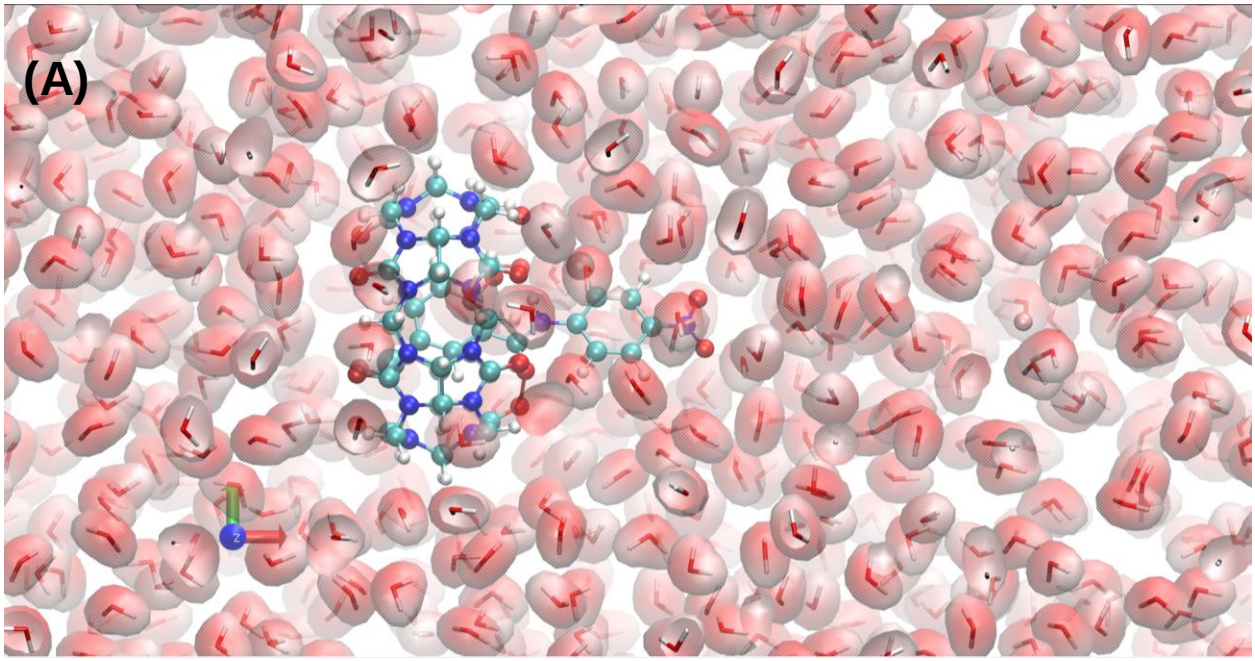
$$E_{restraint} = f_1(1 - e^{f_2(r_0 - r)^2}) \quad \text{(S1)}$$

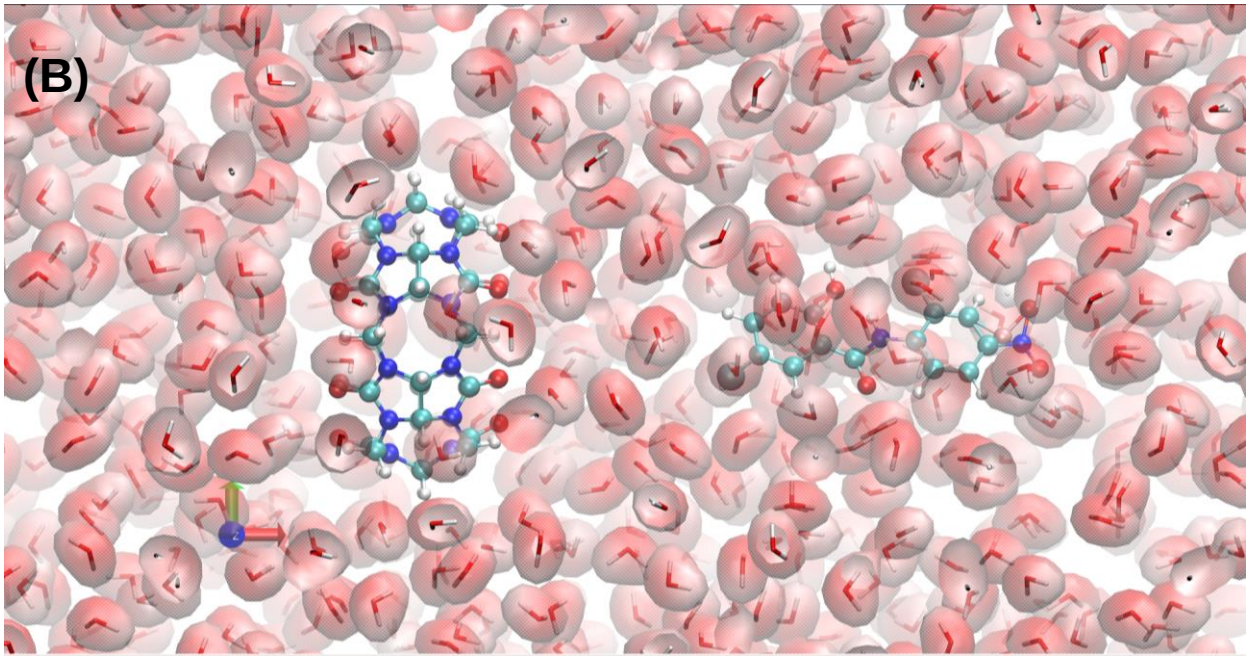
In this expression, f_1 and f_2 are constants; and r_0 is the distance between position A and B during the CMD step at finite temperature of 300K under NVT ensemble. The value of the distance between two sites r is modified to sample the pathway between two local minima initially separated by the distance r_0. The effect of pH variation was investigated by adding H^+ ions into the aqueous solution. In order to maintain the charge neutrality of each system, Cl^- ions were added to the simulation box. We considered three different pHs equal to 1, 3, 7. The acidic environments are provided by ionic species by directly adding 16 H^+ at pH 1, 12 H^+ at pH 3. Each solvated complex was subjected to energy minimization using the conjugate gradient algorithm (CG) prior to starting the MD simulation.

Our initial inspection of the trajectory during the MD simulation shows stable binding of CB6 at state A of the host-guest complex. Actually, our observation shows that irrespective of the deformation of CB6 molecule, almost no movement of CB6 is taking place during the 2 ns of our NPT simulation. This indicates there is a high energy barrier for the migration of Nic throughout CB6 complex. Figure S10 C illustrates the potential energy changes as a function of distance for the CB6 Nic complex.

Our energy calculations demonstrates that whereas the release of Nic molecules consists of a huge amount of energy barrier of 37 eV in the pure water, increasing the concentration of H^+ can decrease the barrier to 12 eV and 7 eV in pH 5 and 3, respectively. It should be noted that the added H^+ ions were diffusing towards the vicinity of the OH^- site in the Nic molecules. The inspection of the trajectories associated with the CMD simulations shows that there are two mechanisms involved to assist the Nic molecule to be extracted from the cavity of the host. First, the protonation of OH^- group sitting in the conjunction of two

aromatic loops of the guest molecule which is facilitated by increasing the concentration of H^+ ions. Due to higher pKa of OH^- in the phenol group, 16.7, the protonation likely occurs near site A. Second, increasing of possible reaction sites for hydrogen bond formation along the host molecule can assist the motion of Nic to release from the CB6 guest by breaking the symmetry of host-guest interaction.




(B)

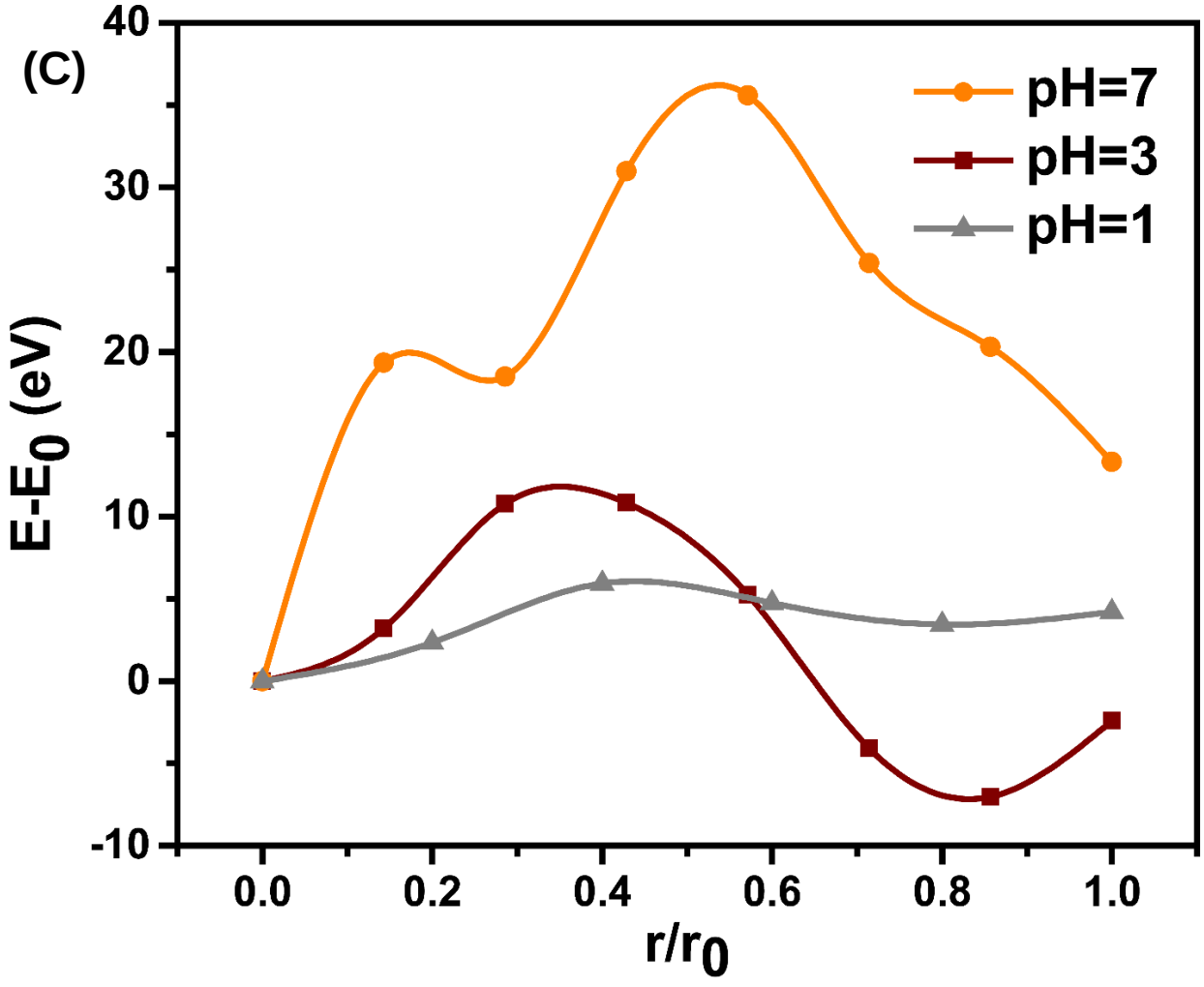


Figure S12. (A) and (B) two configurations considered for carrying in-silico studies in aqueous media, (C) Potential energy landscape for extraction of Nic from CB6 guest molecule under different pH conditions. The barrier energy decreases by decreasing pH.

METHODS AND EXPERIMENTS

Materials

Food grade agave nectar (HoneyTree's® Organic Agave Nectar, Onsted, MI) was purchased from local store. Cucurbit[6]uril (CB[6]) hydrate (CAS Number 80262-44-8, Empirical Formula $C_{36}H_{36}N_{24}O_{12} \cdot xH_2O$) and niclosamide, 98% (HPLC) (2',5-Dichloro-4'-nitrosalicylanilide, CAS Number 50-65-7) were supplied from Sigma Aldrich (St. Louis, MO, USA) and AKA Scientific Inc. (Union City, CA, USA), respectively. Nanopure water (0.2×10^{-6} M, 18 MΩ cm) was used throughout the experiment unless otherwise stated. For biological assessments, human breast cancer MCF-7 cell line was obtained from American Type Culture Collection (ATCC) and was cultured using the prescribed instructions from the ATCC. High Glucose Dulbecco's Modified Eagle's Medium (DMEM; Sigma) was supplemented with 10% fetal bovine serum (FBS (Seradigm, US)) and 1% Penicillin Streptomycin (PenStrep) (Lonza). Trypsin (EDTA 0.02%, dextrose 0.05%, and trypsin 0.1%) in Dulbecco's phosphate buffer saline (DPBS, Gibco) was prepared. Tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide was obtained from Sigma. ^{1}H NMR measurements were carried out in DMSO-d6 (Cambridge Isotope Laboratories, Inc., MA, USA) as the solvent. Other reagents were obtained from Aldrich Chemical Co. (St. Louis, MO) and were used without further purifications. Athymic mice were purchased from Charles River Laboratories International, Inc., USA. All the antibodies were purchased from Molecular probes, Thermo fisher scientific, USA.

HOST-GUEST COMPLEX FORMATION BETWEEN CB6 AND NIC

Computational method

The starting geometries of the studied CB6 Nic complexes were generated by the manual insertion of the guest molecule (Nic) into the desired position of the host molecule (CB). Prior to the molecular dynamics simulations, the initial configurations of CB6,7 and Nic, borrowed from protein data bank (PDB), were minimized using ReaxFF. In order to determine the stability of Nic CB6 complexes, the binding energy of n Nic: m CB6 complexes was calculated based on **Equation S2**:

$$\mathrm{E}_f(n\mathrm{Nic}:m\mathrm{CB6}) = \frac{1}{\mathrm{m}}(\mathrm{E}(n\mathrm{Nic}:m\mathrm{CB6}) - n\mathrm{E}(\mathrm{Nic}) - m\mathrm{E}(\mathrm{CB6})) \quad \text{(S2)}$$

The negative sign of binding energies signifies that the insertion of the Nic molecules into the host molecule is energetically favorable.

Each solvated complex was subjected to energy minimization using the conjugate gradient algorithm prior to starting the MD simulation. The minimized complex was then equilibrated in 300 K for 60 ps, followed by a 500 ps equilibration step at 300 K at 1 atm. To account for the energetics of Nic release in water, the bond-restraint sampling was repeated through dragging of the Nic molecules away from CB6 host. Subsequently, the production runs were carried out for 1 ns at 300 K and 1.0 atm with a 0.1-fs time step under NVT ensemble (Brendsen thermostat with T_{damp}=100.0 fs). The Materials Studio-8 program was used to visualize the structures.

In addition, we computed the enthalpy of binding as the difference of the energy of complex and the individual molecules, where the initial states are obtained from series of energy minimization steps consisting of a conjugant gradient (CG) geometry optimization using ReaxFF with a convergence criterion of 0.5 kcal/mol between subsequent CG steps.

Stoichiometry of the interaction

For the determination of the stoichiometry of the complexation event, Job's plot was constructed by plotting the amide I peak wavenumber versus the mole fraction. The mole fraction of CB6 was varied between 0 and 1 while keeping the total molar concentration constant at 0.5 mM. A total of 7 points were acquired for making the Job's plot. The minimum was taken as the Stoichiometric ratio.

^{1}H-NMR titrations

^{1}H NMR measurements were carried out on Varian VXR 500 (Varian, Inc., Palo Alto, CA) spectrometer operating at 500 MHz equipped with 5mm Nalorac QUAD probe. Chemical shifts were reported in ppm and referenced to the solvent proton impurities. DMSO-d6 was used as the deuterated solvent for all samples. 700 µl of Nic (2 mM) was transferred to the NMR tube. The temperature was raised to 37 ºC so as to ensure accurate shimming. A total of 128 acquisitions were made. Then 10 µl of titrant containing CB (30 mM) and Nic (2 mM) was added at each step and the acquisition was made. The data was processed and analyzed with MestRenova™ 8.1 software (Mestrelab Research SL; Santiago de Compostela, Spain).

Fluorescence spectroscopy titrations

2D excitation, emission Fluorescence spectra were collected using a Horiba Aqualog Scanning Spectrofluorometer (Horiba scientific, Edison, NJ). 3000 µL of Nic (0.04 mM) was transferred to the cuvette and 2D excitation (240–700 nm) and emission (240-800 nm) contour was obtained at 3 nm resolution. Contribution of solvent was subtracted using nanopure water as the blank sample. After each measurement, the first- order Rayleigh scattering was corrected and all spectra were normalized to 1 $mg.l^{-1}$ quinine sulfate. Subsequently 30 µL of titrant containing Nic (0.04 mM) and CB[6] (0.6 mM) was added and mixed.

NANOPARTICLES SYNTHESIS AND CHARACTERIZATIONS

Carbon nanoparticles decorated with cucurbit[6]uril synthesis

Pristine carbon nanoparticles (CNP) were carbonized by facile hydrothermal method using commercially available food grade agave nectar as the carbohydrate source (batch composition; 47-56% fructose and 16-20% glucose, rest is mixture of other sugars and water). Agave nectar dispersed in water (0.2 $mg.ml^{-1}$) was slowly heated on a hotplate at 300 ºC (Corning®) so as to evaporate water and the product visually changed color from amber to dark brown. Subsequently, the burnt residue was resuspended in water and probe sonicated (Q700, Qsonica Sonicators, Newtown, CT) in the ice bath for 5 min at Pulsed Amp, 1; on, 5 s;

off, 3 s. To obtain a narrow size distribution, the as synthesized nanoparticles were purified by dialysis cassettes using cellulosic membrane (10K MWCO, 3ml, Thermo Scientific, IL, USA) against nanopure (0.2×10^{-6} m) water for a prolonged period of time. Subsequently, the content underwent two steps of additional filtration (Acrodisc syringe) with mesh size of 0.45 μm and 0.22 μm, respectively.

Two methods of synthesis were applied for CNPs decorated with CB6 preparation. In the first method, CB6 was introduced on the surface of CNP *via* vigorous stirring of the respective concentrations of CB6 for 5 min using vortex machine. These particles were labeled CB CNP post functionalized henceforth. In the second method, one pot nucleation of CB6 CNP was carried out. Briefly, the mixture of agave nectar and CB6 suspended in water at the desired concentration was heated on the hot plate and similar steps as for CNP preparation was followed. These particles were denoted as CB6 CNP prefunctionalized. Comprehensive physicochemical characterizations of the prepared nanoparticles were carried out.

In order to optimize the size of the nanoparticles and achieve a single layer coating of CB6 on CNPs, the concentration of CB6 was varied between 10^{-6}-10^{-1} mM with 10 times dilution in each step while keeping the amount of CNP unchanged. Subsequently, the hydrodynamic size of the particles was measured and plot of size vs. concentration was made. The optimized concentration was used for the prefunctionalized samples and the rest of experiments.

Preparation of CB6 CNP Nic

A stock solution of Nic was prepared in ethanol (2 mM) and then diluted with water to reach the desired concentration depending on the experiment. The drug was loaded on the CB6 CNP (pre/ post functionalized) using simple mixing method for 5 min.

Dynamic light scattering experiment

The particles were mildly vortexed prior to size determination. The hydrodynamic size and the distribution of the nanoparticles were determined through dynamic light scattering measurements on Malvern Zetasizer ZS90 instrument (Malvern Instruments Ltd, United Kingdom) at fixed angle of 90º. A photomultiplier aperture of 400 mm was used and the incident laser power was adjusted so as to obtain a photon counting rate between 200 and 300 kcps. The measurements whose measured and calculated baselines of the intensity autocorrelation function fell in +0.1% range were used to determine nanoparticle hydrodynamic diameter. The mean hydrodynamic diameter (Z-average) values were obtained based on the correlation function. All measurements were carried out in triplet of twelve consecutive measurements.

Transmission electron microscopy (TEM)

Prior to sample preparation for TEM imaging, the nanoparticles were mildly vortexed. 10 µL of sample was drop cast on 200-mesh copper grid. After 1 min, excess fluid was absorbed by lint free filter paper. The transmission electron micrographs were acquired on a JEOL 2100 Cryo TEM machine (Tokyo, Japan) and imaged by Gatan UltraScan 2k × 2k CCD

camera. The same concentration of CB6 CNP prefunctionalized and CB6 CNP postfunctionalized samples were used. The anhydrous diameter was determined by making 100 random measurements of nanoparticles on image J software (NIH, Bethesda, MD) and was reported as mean± standard deviation.

Atomic force microscopy (AFM)

An aliquot of nanoparticle suspension was placed on the top of freshly cleaved mica sheet attached to a stainless steel disc to minimize unwanted strains. Next, the specimens were vacuum dried overnight. AFM micrographs were obtained in the tapping mode on Asylum Cypher (Asylum Research, Santa Barbara, CA) device.

Size stability of CB6 CNP Nic

Stability of the formulation was monitored over a period of 30 days by evaluating the hydrodynamic diameters at different time points. The nanoparticles were incubated at three different conditions namely, aqueous solution with pH=7.4, aqueous solution with pH 4.5 (to mimic cancer cell environment) and aqueous solution containing 10% FBS. The samples were kept in water bath cycled to 37 ºC and were removed for DLS reading at the corresponding time intervals.

Electrophoretic zeta potential measurement

Zeta potential ζ values were determined using a Malvern Zetasizer (Malvern Instruments Ltd, United Kingdom) of nano series. The experiment was performed at 25 ºC

and at pH=7 at the light scattering mode following solution equilibration at 25 °C. Calculation of ζ from the measured nanoparticle electrophoretic mobility (μ) employed the Smoluchowski equation: $\mu = {}^{\varepsilon\xi}/_{\eta}$ where ε and η are the dielectric constant and the absolute viscosity of the medium, respectively. Measurements of ζ were reproducible to within ±2 mV of the mean value given by 3 determinations of 15 data accumulations.

UV-Vis spectroscopy

Absorption spectra from different samples were recorded using GENESYS 10 UV-Vis spectrometer (Thermo Scientific, MA, USA). The samples were scanned in 220-800 nm range and the scan resolution was varied from 0.1-0.5 nm based on the requirements of the experiment. Experiments were carried out in 1 cm plastic cuvettes with the covering wavelength range between 230-900 nm. Throughout all the experiments, the samples were diluted such that the solutions obeyed the Beer Lambert law to maintain highest accuracy. Concentration of a component of interest was determined based on carefully calibrated curves obtained by reading serial dilutions of the samples.

Fourier transform infrared (FT-IR) spectroscopy

As-synthesized nanoparticles were repeatedly applied to MirrIR IR-reflective glass slides (Kevley Technologies, Chesterland, OH, USA) to acquire IR spectra using a PerkinElmer Spotlight 400 (PerkinElmer, Waltham, MA, USA) equipped with a thermal source, and a raster-scanning linear array detector. Spectra were collected using a 1 $cm.s^{-1}$

mirror speed for acquisition, and zero padding was not used. Background scans were taken at 8 cm^{-1} spectral resolution; 150 × 150 μm images were collected at 8 cm^{-1} spectral resolutions with 8 scans per pixel and a 6.25 × 6.25 μm pixel size. Data were atmosphere corrected on the spotlight and further processing was done using Matlab R2013 software.

Raman spectroscopy

Raman spectra were acquired on the dried samples in reflection mode (LabRAM Horiba). The excitation wavelength for all measurements was 532 nm and the power was set to 25 mW at the sample with a 10 s acquisition time. The Raman shift from 400 to 4,000 cm^{-1} was collected at 8 cm^{-1} spectral resolution. Laser light was focused through a 100×, NA 0.8 objective into the sample plane and the scattering was collected in the reflection geometry using the spectrograph coupled with an Andor Newton back-illuminated EMCCD camera (LAB Ram, Horiba).

***In vitro* dissolution experiment of CB6 CNP Nic**

The *in vitro* drug release profile were monitored under various physiologically relevant conditions and prolonged for 120 hours. Specifically, two conditions were adopted namely phosphate buffer saline (pH=7.4) and acetate buffer solution (pH=4.6) at 37 °C. 500 μl of CB6 (10^{-3} mM) CNP Nic (0.1 mM) was transferred into Slide-A-lyzer dialysis devices (molecular weight cut off: 10K, 0.5 ml, Thermo Scientific, IL, USA) and subjected to dialysis against 1 L of the media to simulate sink condition. The beaker was magnetically stirred (120 rpm) to keep the conditions homogenous across the solution. At the indicated time points, one

sample was recovered and diluted with water (60 µl sample for 940 µl of water) for quantitative analysis. The percentage of the released drug was determined using characteristic absorbance of Nic at 377 nm.

IN VITRO BIOLOGICAL STUDIES

Cancer cell culture

Cell toxicity assay was performed on human breast cancer ER (+) MCF-7 (ATCC) cells. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) in T25 culture flasks (Cellstar; Germany) and were incubated at 37 °C in a 99% humidified atmosphere containing 5% CO_2. Cells were regularly passaged by trypsinization with 0.1% trypsin (EDTA 0.02%, dextrose 0.05%, and trypsin 0.1%) in DPBS (pH 7.4). Nonsynchronized cells were used for the experiments.

Cell toxicity assay

The cell toxicity assay measures the potency of the drug in inhibiting the cell growth and compared by calculating IC_{50} (inhibitory concentration for 50% cell population). MCF-7 cells were seeded in the 96- well- tissue culture polystyrene plates with the density of 10^4 cells per well. After 24 h the cells were treated with the samples prepared in growth medium at various concentrations ranging from 1.5-50 µM. After 44 h of incubation, cells were imaged for bright field imaging to visualize the effect of treatments on cell growth density and changes in cellular morphology. It was followed by the addition of a 20 µl (5 $mg.ml^{-1}$) solution of MTT to the growth medium while growing the cells. MTT turns into purple

formazan after reduction by mitochondrial enzymes in the living cells. MTT added cells were incubated for 4 h at 37 °C under 5% CO_2 atmosphere. The solution was removed and replaced by 200 µl of dimethyl sulfoxide (DMSO) added to each well and incubated for another 5 min. The solution was mixed by pipette in order to completely dissolve the dark blue crystals from the samples. Absorption was read on multi-well plate reader machine (BioTek Synergy HT, US) using the standard wavelengths of 592 nm. An average of the triplicate wells were calculated and normalized by dividing to the value of control well, the standard error mean was calculated for each sample using Graph pad prism 6.0 software.

Cell mechanistic studies

Cells were treated with Nic formulations followed by flow assisted cell sorting (FACS) analysis on propidium iodide (PI) stained treated and untreated cells. Results were analyzed to calculate the apoptotic cell population. MCF-7 cells (0.4 x 10^6 per well) were plated in 6 well plates and grown till it achieved ~80 % confluence. After ~24h of incubation, cells were treated with 50 µM of free Nic or loaded with CB6 and CB6 CNP. At the end of 72h time point, cells were imaged under bright field microscope for cell growth density and morphological determination. Cells were trypsinized and collected in 100 µl of reconstituted medium (10% FBS in DMEM). Cell pellets were fixed with homogenization in chilled ethanol while vortexing. Fixed cells were stored at -20 °C for >12h. At the end of the incubation, cells were washed with DPBS at least two times and incubated with RNase A (1 µg/mL) at 37 °C for >12h. Cells were incubated with PI (2 µg/mL) for 30 min before

scanning on FACS machine. Cells were treated in triplicate and mixed well before acquiring the FACS data.

Laddering assay for fragmented genomic DNA analysis post apoptosis

DNA fragmentation assay was performed on MCF-7 cells to further probe the cell apoptosis as described earlier. Briefly, cells grown for 24h were treated with Nic formulations for 72h before harvesting in 1 mL of 10% FBS containing culture medium. At the end of the incubation period cells were washed with 1 mL DPBS two times. Harvested cells were spun at 1000 rpm for 2 min. It gave sizable cell pellets. Cell pellets were trypsinized and collected in 400 µL of lysis buffer. DNA extraction was performed using manufacturer's protocol using Thermo Scientific DNA extraction kit. Extracted genomic DNA were washed with 70% EtOH and dissolved in water after air drying. Collected genomic DNA was used to load in 2% agarose gel and ran at 100 mV for 40 min before imaging on Gel doc (Bio-Rad Laboratories, Inc., Hercules, CA).

Cell fixing for imaging and spectroscopy

MCF-7 cell line was allowed to grow in a 6 well plate until reaching ~70% confluence. The cells were then trypisinized and seeded onto sterilized low E-slides (for infrared imaging) and coverslips (for Raman measurements). The cultured cells on the mentioned substrates were incubated with various formulations for 4 hours at 37 °C. Subsequently, they were washed with DPBS and fixed by dehydration using 70% EtOH. To avoid artifacts from DPBS

during acquisition, all the slides were thoroughly washed with deionized water prior to spectroscopy.

IN VIVO EXPERIMENTS

Advanced 3D cell culture techniques can mimic some of the aspects of the *in vivo* tumor environment. To evaluate the efficacy of adopted strategy using CB6 CNP Nic, animal experiments were performed in athymic mice. All experiments were designed to minimize the use of animals. We generated 4 tumors per animal as 3 mice per group. Athymic mice were bought from Charles River Laboratories International, Inc. USA. Upon arrival, athymic mice were allowed 1 week for acclimation. Animals were single-cage housed and had free access to food and water. Animals were housed in Carl R. Woese Institute for Genomic Biology, university of Illinois at Urbana-Champaign.

Injection of MCF-7 (ER(+) human breast cancer cells) in flanks of athymic mice

Animals were anesthetized with isoflurane before injecting the MCF-7 cells suspended in Matrigel (50 %, v/v). Approximately $5x10^6$ MCF-7 human breast cancer cells suspended in 40 μL of Matrigel® were subcutaneously injected into four sites in the flank of each mouse. Mice were monitored during recovery from the anesthesia in a clean cage. MCF-7 tumors grew on the flanks of mice after cell injection. No significant discomfort was noticed in the tumor bearing mice in the time frame of completing the experiment. Mice were monitored daily for the signs of discomfort and behavior change. Mice body weight was measured every week. The change in physiological function or abnormal behavior including shortness of

breath, unsteady gait, abnormal eating behavior, physical abnormalities, rough hair coat due to lack of grooming, or lethargy were reported to division of animal research. Criteria for interventions were set up as tumor increase to 17mm x 17mm. Tumor size was determined by measuring the length and width of the tumor using caliper and then calculating the tumor volume *via* formulae

Tumor volume = $(\text{length}^2 \times \text{width})/2$

Intra-tumoral injections

Animals were followed till tumors grew to a minimum of 4 mm x 4 mm in size before starting the treatment. CB6 Nic was also injected along with DPBS to evaluate the comparative tumor regression efficiency. CB6 Nic and CB6 CNP Nic were prepared at 1.5 mM concentration of Nic. Formulations and DPBS (40 µL) were injected to tumors grown to size of at least 4 mm x 4 mm. Isoflurane-oxygen mixture was used to anesthetize the animals with 3-4% isoflurane gas from a vaporizer and constant anesthesia maintained with 1-2% isoflurane *via* an inlet tube. A second tube was used to remove generated carbon dioxide and excess anesthetic. All the personnel involved wore protective lab coats, face masks, sterile gloves during experimental procedures. A total of 40 µL formulation were injected to each tumor on every 4 day including 16, 20, 24, 28 and 32nd day and followed till 40th day for tumor growth and regression.

Animal dissection, tumor collection, processing, embedding and sectioning

At the end of the experiment, animals were euthanized with CO_2 influx. Animals were dissected to collect tumors and stored in tissue cassettes dipped in 10% formalin before performing the tissue fixation protocol in Leica ASP300 tissue processor. The processing protocol was used including steps of tissue incubation in neutral buffered saline for 45 min, twice with ethanol (70%) for 45 min, ethanol (80%) for 45 min, twice with ethanol (95%) for 45 min, twice with absolute ethanol for 45 min, twice with xylene for 45 min and finally thrice with paraffin wax for 45 min. Processed tumors were embedded in paraffin wax melted at 65 °C using metal cast. Embedded tumor blocks were clamped in microtome (Leica) and sectioned at 6 μm thickness.

Hematoxylin and eosin (H&E) staining

Paraffin-embedded sections (7 μm) were subjected to hematoxylin and eosin (H&E) staining following the standard protocol supplied at core facility IGB. In brief, sections were processed by deparaffinizing sections with 3 changes of xylene for 5 minutes each; re-hydrating in 3 changes of absolute alcohol for 5 minutes each; 95% alcohol for 3 minutes and 70% alcohol for 3 minutes each; washing briefly in distilled water; staining in Mayer hematoxylin solution for 3 minutes; washing in warm running tap water for 5 minutes; rinsing in distilled water; rinsing in 95% alcohol, 10 dips; counterstaining in eosin-phloxine B solution (or eosin Y solution) for 3 minute; dehydration through 95% alcohol, 2 changes of absolute alcohol, 5 minutes each; clearing in 2 changes of xylene, 5 minutes each; mounting

with xylene based mounting medium. The morphological changes of H&E-stained tissue with each fixation were analyzed at magnification ×40.

Immunolabelling of tissue sections for pSTAT3 and β-actin

To image the extent of pSTAT3 protein expression in treated and untreated tumors, immunolabelling was performed on paraffin-embedded sections (7 μm). Immunolabelling was performed by following standard protocol supplied by core facility IGB. In brief, sections were processed by de-waxing of slides in histoclear (3x) for 5 min each, 100% EtOH for 5 min, 70:30 EtOH/PBS for 5 min, 50:50 EtOH/PBS for 5 min, 25:75 EtOH/PBS for 5 min and finally with PBS (3x) for 5 min each. It was followed by citrate buffer antigen retrieval buffer protocol where sodium citrate buffer was prepared using tri-sodium citrate (dehydrate), 10 mM (pH 6 made up by using 1N HCl) mixed with citrate.

Enzymatic degradation of CB6 CNP Nic

Five suspensions of CB6 CNP Nic (with carbon content of 10 $mg.ml^{-1}$) were prepared in Dulbecco's phosphate buffered solution and 200 μM of hydrogen peroxide. Subsequently, 100 μg of human myeloperoxidase (hMPO) enzyme (activity > 50 $units.mg^{-1}$ protein) was added to four of the five samples and statically incubated at 37 ˚C in the dark. Hydrogen peroxide was replenished every 5 h to sustain the activity of enzyme. To quench the enzyme activity, samples were transferred to 4 ˚C after 6, 12, 24 and 168 h. The collected samples were drop cast on glass slides for Raman analysis (120 to 2700 nm at 2% laser power for 20 seconds). All Raman measurements were taken on Nanophoton RAMAN 11 laser Raman

microscope (Osaka, Japan) with a 532 nm wavelength laser for 20 seconds at 2% laser power using a 20x objective. For each spectrum a grating (600 l mm^{-1}) scan was taken over the range 120 - 2700 cm^{-1}. An average of 20 spectra were recorded and averaged for each measurement sample.