**Supplementary Information**

# Enriched Inhibition of Cancer and Stem-like Cancer Cells via STAT-3 Modulating Niclocelles

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**Table S1A.** Composition of optimized niclocelle and nanocelle and their stability patterns.

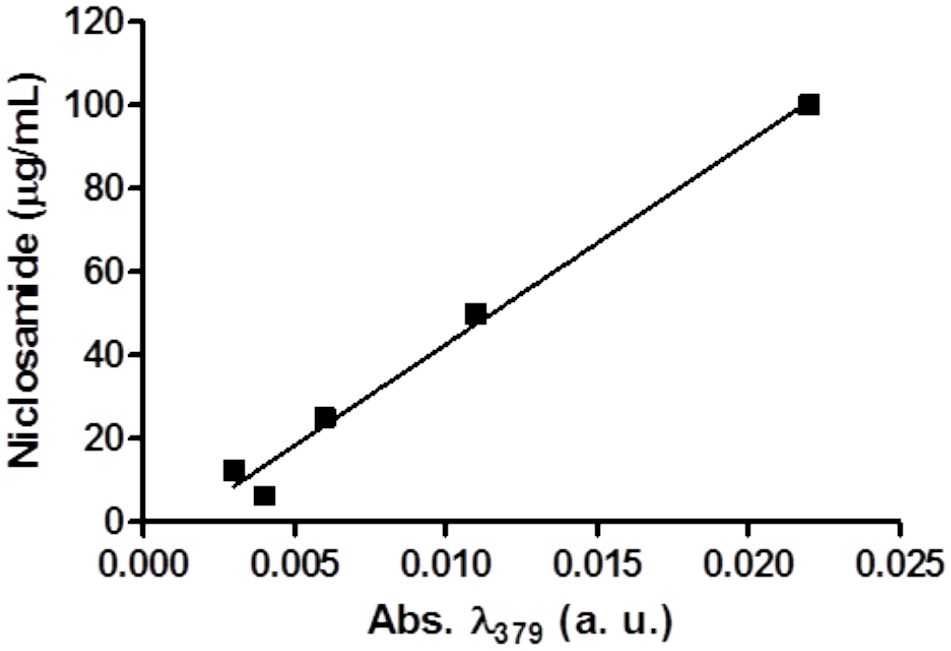
| | Sample | PEGCE (mg/mL) | PS-b-PAA (2 mg/mL) THF (µL) | Niclosamide (µg/mL) | Hydrodynamic diameter (number avg. ,nm) | | | Stabile/unstable |
|---|---|---|---|---|---|---|---|---|
| 1 | PEGCE | 2 | - | - | 10 | 10 | 9 | S |
| 2 | Ps-b-PAA | - | 250 | - | - | - | - | - |
| 3 | Niclosamide | - | - | 100 | - | - | - | - |
| 4 | Nanocelle-I | 2 | 500 | - | | | | |
| 5 | Niclocelle-I | 2 | 500 | 100 | 498 | 495 | 477 | S |
| 7 | Nanocelle-II | 1 | 500 | - | 302 | 308 | - | US |
| 8 | Niclocelle-II | 1 | 500 | 100 | 25 | 43 | - | US |
| 9 | Nanocelle-III | 1 | 50 | - | 64 | 47 | 74 | S |
| 10 | Niclocelle-III | 1 | 50 | 100 | 19 | 25 | 15 | US |
| 11 | Nanocelle-IV | 1 | 100 | - | 57 | 33 | 57 | S |
| 12 | Niclocelle-IV | 1 | 100 | 100 | - | - | - | US |
| 13 | Nanocelle-V | 0.5 | 100 | - | - | - | - | US |
| 14 | Niclocelle-V | 0.5 | 100 | 100 | 294 | 282 | 290 | US |
| 15 | **Nanocelle** | **0.5** | **250** | **-** | **93** | **99** | **89** | **S** |
| 16 | **Niclocelle** | **0.5** | **250** | **100** | **69** | **70** | **-** | **S** |
| 17 | **Post-Niclocelle** | **0.5** | **250** | **100** | **32** | **24** | **53** | **US** |
| 19 | Nanocelle-VII | 0.5 | 500 | - | 121 | 128 | 160 | S |
| 20 | Niclocelle-VII | 0.5 | 500 | 100 | 29 | 46 | 47 | S |

PEGCE: Polyethylene glycol cetylether; THF: Tetrahydrofuran; Ps-b-PAA: Polystyrene-block-polyacrylic acid; S = Stable and US: unstable. Candidates marked in red were used for further biological studies in this work.

**Table S1B.** DLS values of niclocelle and nanocelle with volume and intensity average.

| | Sample | Hydrodynamic diameter (volume avg., nm) | | | Hydrodynamic diameter (Intensity avg. ,nm) | | | PDI |
|---|---|---|---|---|---|---|---|---|
| 1 | PEGCE | 9 | 9 | 8 | - | - | - | 0.5 |
| 2 | Ps-b-PAA | - | - | - | - | - | - | - |
| 3 | Niclosamide | - | - | - | - | - | - | - |
| 4 | Nanocelle-I | - | - | - | - | - | - | - |
| 5 | Niclocelle-I | 306 | 305 | 307 | - | - | - | 0.3 |
| 7 | Nanocelle-II | - | - | - | - | - | - | - |
| 8 | Niclocelle-II | - | 138 | 167 | 202 | - | - | 0.4 |
| 0.49 | Nanocelle-III | - | - | 54 | - | 50 | 54 | 0.3 |
| 10 | Niclocelle-III | 25 | 43 | - | - | - | - | 0.4 |
| 11 | Nanocelle-IV | 64 | 47 | 74 | - | - | - | 0.7 |
| 12 | Niclocelle-IV | - | - | - | - | - | - | - |
| 13 | Nanocelle-V | - | - | - | - | - | - | - |
| 14 | Niclocelle-V | - | 290 | 282 | 262 | 294 | - | 0.7 |
| 15 | **Nanocelle** | **99** | **106** | **107** | **120** | **94** | **-** | **0.2** |
| 16 | **Niclocelle** | **70** | **70** | **69** | **69** | **69** | **68** | **0.2** |
| 17 | **Post-Niclocelle** | **115** | **79** | **-** | **91** | **110** | **80** | **0.5** |
| 19 | Nanocelle-VII | 120 | 128 | - | - | - | - | 0.2 |
| 20 | Niclocelle-VII | 27 | 45 | 48 | - | - | - | 0.8 |

(a)




(b)

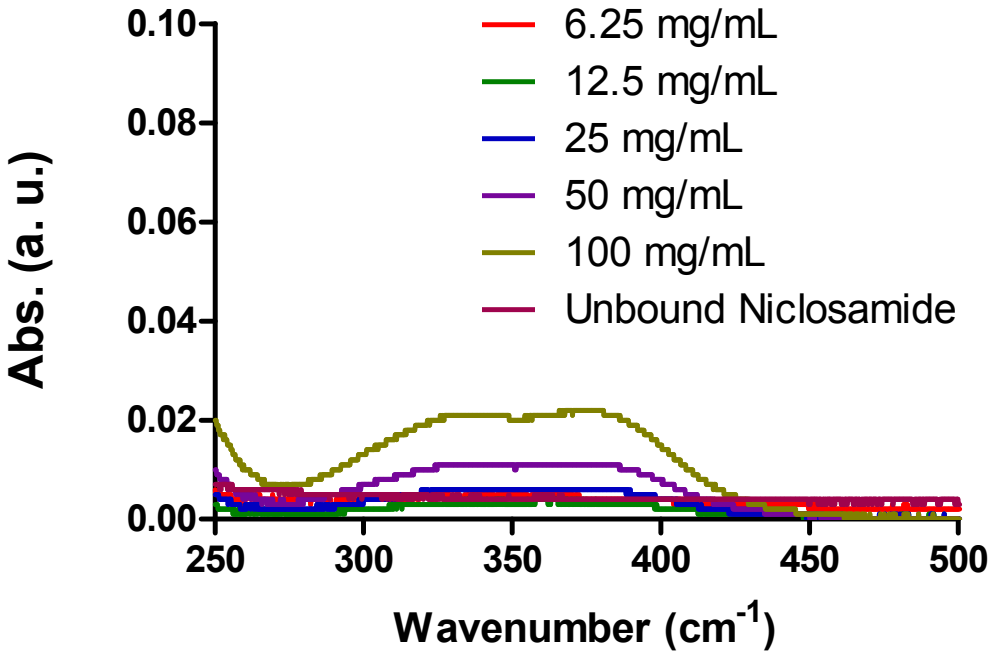



Figure S1. Calculation of niclosamide loading efficiency in Niclocelles. (a) calibration curve from known niclosamide amounts and (b) UV-vis spectra from known and unbound amount of niclosamide. The amount of unbound niclosamide was found to be 13.1 µg per ml of suspension while the loading percentage was calculated as 86.9%.

Table S2. Wavenumbers from various vibrational modes of major individual components of niclocelle and comparison with niclocelle.

| **Niclosamide** | **Functional group** | **C=C** | **C=O (Amide)** | **N-H** | **C-N** | **NO2** | **C-O** | **C-OH** |
|---|---|---|---|---|---|---|---|---|
| | Wave No | 1606 | 1648 | 1560 | 1284 | 1518 | 1216 | 1192 |
| | vibration | C=C stretch | C=O Stretch | N-H bend | C-N stretch | NO2 stretch | C-O stretch | C-OH stretch |
| PS-b-PAA | Functional group | C=C (Vinyl) | C=O (Acid) | C=C (Aromatic) | C=C (Aromatic) | | | |
| | Wave No | 1602 | 1702 | 1492 | 1450 | | | |
| | vibration | C=C stretch | C=O Stretch | C=C stretch | C=C stretch | | | |
| Niclocelle | C=C | C=O (Amide) | N-H | C-N | NO2 | C-O | | |
| | 1590 | 1646 | 1580 | 1280 | 1492 | 1240 | | |
| | C=C Stretch | C=O Stretch | N-H bend | C-N Stretch | NO2 Stretch | C-O Stretch | | |

Table S3. Niclosamide release % at different time points

| Time point | % Niclosamide release |
|---|---|
| 3h | 0 |
| 6h | 2 |
| 12h | 15 |
| 24h | 41 |
| 48h | 61 |
| 72h | 80 |
| 96h | 82 |

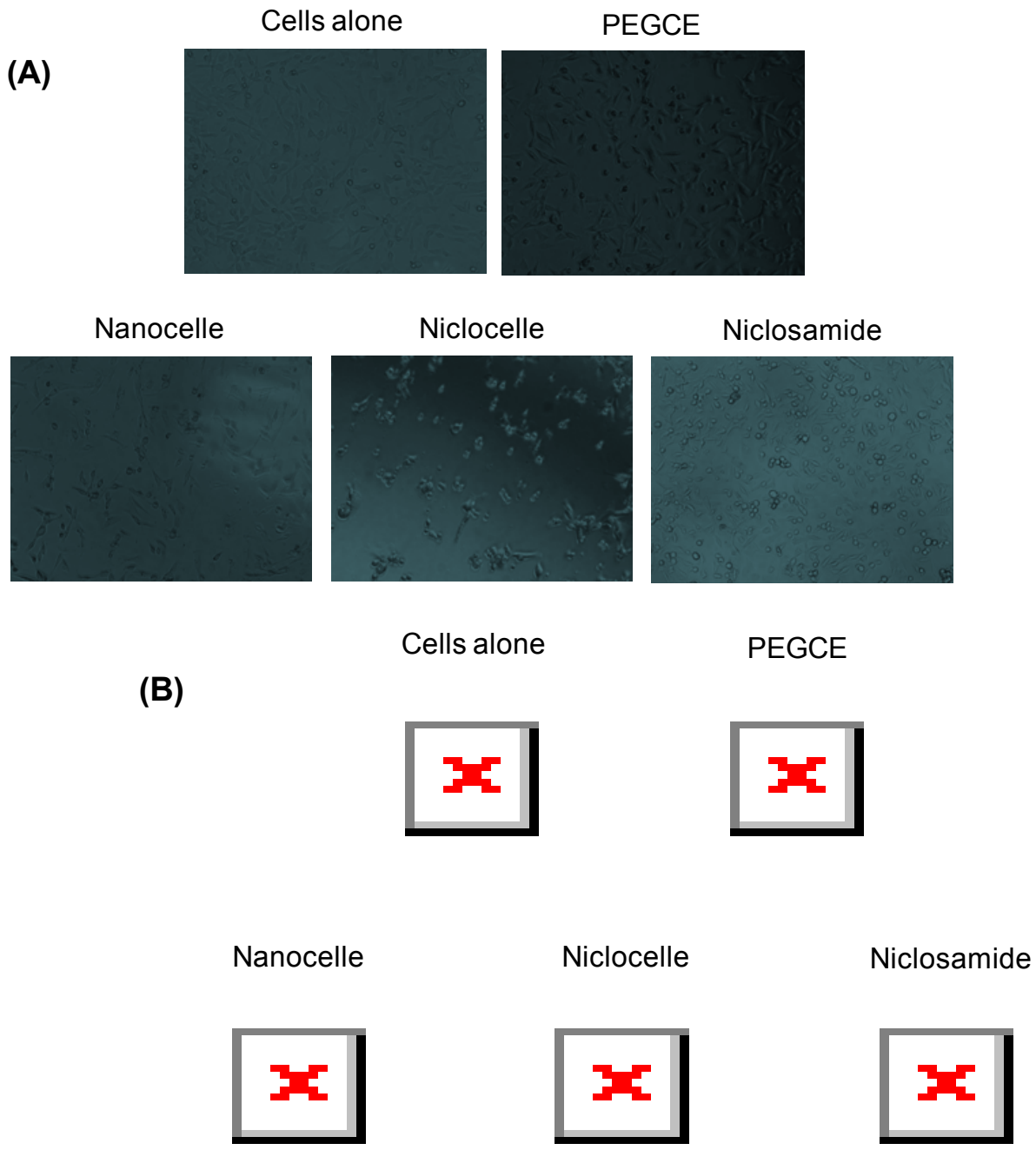



**Figure S2.** Bright field images of (A) MDA-MB231 and (B) MCF-7 cells treated with different formulations. Niclosamide and Niclocelle were used at 20 μM concentration while Nanocelle and polyethylene glycol cetylether (PEGCE) were used at same volume.

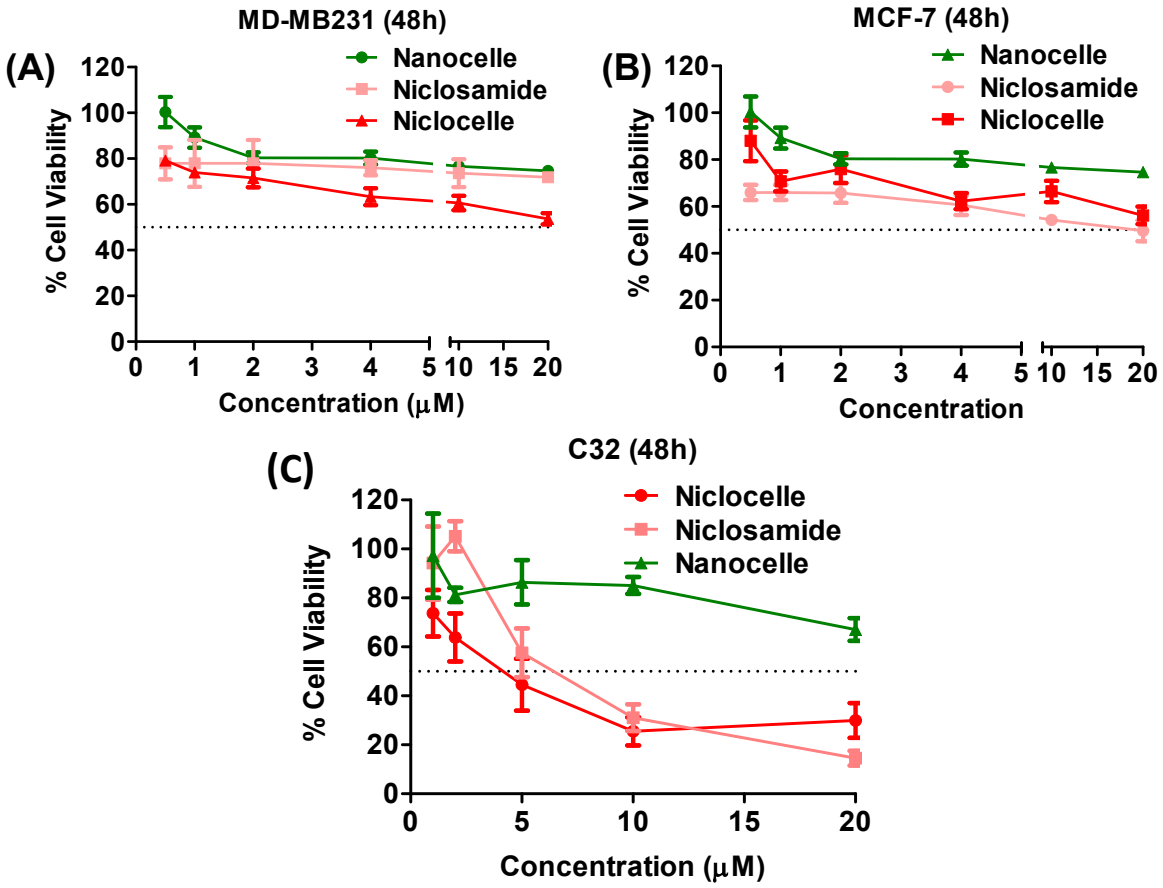



**Figure S3.** MTT assay performed in MDA-MB231, MCF-7 and C32 cells with nanocelle, niclosamide and niclocelle after 48h of incubation at niclosamide concentration ranging from 0.6125 to 20 µM.

## Experimental

### Materials

Polyethylene glycol cetyl ether and poly(styrene)-block-poly(acrylic acid) were obtained from Sigma Life Sciences (St. Louis, MO, U.S.A). Tetrahydrofuran was obtained from Avantor Performance Materials (Center Valley, PA, U.S.A.). The hydrodynamic diameter was measured

on Malvern Zetasizer machine equipped with 633 nm laser. UV-Vis spectra were recorded on Genesys 10S UV-Vis Spectrophotometer machine. Zetapotential measurement was performed on Malvern Zetasizer instrument. Atomic force microscopy was performed on MFP-3D AFM from Asylum Research using Igor Pro software. The TEM images were acquired on JEOL 2100 Cryo TEM machine and imaged by Gatan UltraScan 2kx2k CCD. The XRD data was collected on instrument Siemens-Bruker D5000 diffractometer and analyzed using software Jade X-ray analysis. Flow assisted cell sorting was performed on an iCyt Reflection machine from iCyt Mission Technology equipped with software Win List 3D.

**Preparation of Niclocelles and Nanocelles**

Rigid core polymeric micelles (Nanocelle) were prepared by solvent evaporation method following earlier reported procedure.[1] In brief, polyethylene glycol cetyl ether (0.5, 1 and 2 mg) was melted at 65 °C for 5 minutes, followed by addition of 1 mL of autoclaved water drop-wise. The prepared miceller suspension was left to stir for next 20 minutes at 1150 rpm. Poly(styrene)-block-poly(acrylic acid) (PS-b-PAA)/THF solution (125, 250, 500 µL; 2 milligrams/mL) was added drop-wise (approximately 1 drop per 10 seconds) to the stirring miceller suspension. Niclosamide was incorporated to the preparations by two different methodologies. The 10 µL of niclosamide ethanol solution (10 mg/mL) was added to the preparations either before or after addition of PS-b-PAA and called Niclocelle and Post-Niclocelle. The organic solvent was evaporated under stirring condition for >12h. Total volume was made up to 1 mL with added autoclaved water. The suspension was further allowed to stir for 10 minutes. Prepared Niclocelles and Post-Niclocelle were stored at 4 °C overnight for curing the core of the particle and the characterized the cured particles with various physico-chemical techniques.

**Dynamic Light Scattering**

Dialysis (MWCO 20 kDa dialysis tubing, Spectrum Laboratories, Rancho Dominguez, CA) of were performed nanoparticle suspensions into deionized water (0.2 μM) before performing characterization measurements and applications. A nano series Zetasizer instrument was used to measure the hydrodynamic diameter of prepared formulations, Niclocelle and PostNiclocelle along with control nanocelle particles. The solution was stored at 4°C and mildly vortexed before acquiring particle size again and/or other experiments. Malvern Zetasizer ZS90 particle size analyzer was used to determine hydrodynamic diameters. Scattered light was collected at a fixed angle of 90°. A photomultiplier aperture of 400 mm was used, and the incident laser power was adjusted to obtain a photon counting rate between 200 and 300 kcps. Only measurements for which the measured and calculated baselines of the intensity autocorrelation function agreed to within +0.1% were used to calculate nanoparticle hydrodynamic diameter values. All determinations were made in multiples of 3 consecutive measurements with more than 10 runs each time. [2]

**Zeta Potential Measurements**

Zeta potential measurement was performed on samples Niclocelle and nanocelles, using a Malvern zetasizer of nano series. All the experiments were performed at 25 $^{o}$C. Data were acquired in the phase analysis light scattering (PALS) mode following solution equilibration at 25 °C. Calculation of ζ from the measured nanoparticle electrophoretic mobility (μ) employed the Smoluchowski equation: $\mu = \varepsilon\zeta/\eta$, 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. [2]

**UV-Vis Spectroscopy**

UV-Vis spectroscopy was performed on Niclocelle and nanocelle samples along with free niclosamide, using a Genesys 10S UV-Vis Spectrophotometer. Aqueous samples were scanned for niclosamide concentration of 10 μM in free or nanoparticle loaded form in aqueous solution. Niclosamide was also scanned for absorbance spectra in EtOH, where solubility of niclosamide was much better compare to aqueous medium.[3]

**Cast-film X-Ray Diffraction Measurement**

The lamellar arrangements and variations in inter-layer spacing of polymeric shell of Niclocelle and nanocelles were determined by X-ray diffraction (XRD) measurement. The aqueous aggregates of each formulation were placed on a pre-cleaned glass plate which, upon air drying, afforded a thin film of the formulations on the glass plate. XRD of an individual cast film was performed using the reflection method with a Siemens-Bruker D5000 diffractometer. The X-ray beam was generated with a Cu anode and the Cu-Kα beam of wavelength 1.5418 Å was used for the experiments. Scans were performed for 2Θ range of 20 to 60.[4]

**Atomic Force Microscopy and Transmission Electron Microscopy**

An aliquot of each of Niclocelle and nanocelles was drop-cast onto a newly peeled off mica-sheet and allowed to be air dried for 48 h. Atomic force microscopy (AFM) images were obtained by an Asylum Cypher AFM instrument in a tapping mode. All the formulations were used at same concentration of Nanocelles in blank of drug loaded forms Niclocelle and PostNiclocelle.[1]

An aliquot of Niclocelle and nanocelle was drop-casted onto a carbon coated copper grid (200 mesh size). Samples were stained with 10 µl of uranyl acetate (1%). TEM images were taken on JEOL 2100 Cryo TEM machine and imaged by Gatan UltraScan 2kx2k CCD. The concentrations were used as Niclocelle containing 300 nM of Niclosamide and equal volume of Nanocelle.[1]

**Stability of Niclocelle and Nanocelles**

Stability of formulations incubated with 10% featl bovine serum were determined by evaluating the hydrodynamic diameters at different time points (0, 24, 48 and 72h) using a Malvern Zetasizer ZS90 particle size analyzer.

**Fourier Transform Infrared (FT-IR) Spectroscopy**

As-synthesized particles were repeatedly applied to MirrIR IR-reflective glass slides (Kevley Technologies, Chesterland, Ohio, USA) to acquire IR spectra using a PerkinElmer Spotlight 400 (PerkinElmer, Waltham, Massachusetts, USA) equipped with a thermal source and a raster-scanning linear array detector was used. Spectra were collected using a 1 cm $s^{-1}$ mirror speed for acquisition.

**Loading efficiency**

Niclosamide loading efficiency of Niclocelles was determined by measuring the concentration of unbound niclosamide. Niclocelle suspension was centrifuged at 50,000 rpm for 10 min before collecting the supernatant and acquiring UV-vis spectra. Amount of unbound niclosamide was calculated by performing linear regression analysis along with UV-vis spectra of known

concentrations ranging from 6.25-100 μg/mL. The loading percentage was calculated by applying formula

% Loading efficiency =[(Loaded niclosamide - Unbound niclosamide)/Loaded niclosamide]*100

***In vitro* drug release study**

The *in vitro* drug release profiles of the Niclocelles over 96 h was studied at various time points in DPBS at pH 7.4. Two mL volume of Niclocelle was injected in slide-A-Lyzer® dialysis cassette G2 (10,000 MWCO, Thermo Scientific) and dipped in 200 mL of DPBS. A magnetic stir was put in beaker for continuous stirring at 37 °C. The drug release pattern was observed with an initial release of ~`41% in the first 12 h by a sustained release of the drug leading to total release of about 81% in 96 h of observation.

**Gel electrophoresis**

In order to find out the interaction of Niclocelle and nanocelle with pDNA gel electrophoresis was performed. Formulations were incubated with pDNA (w/w ratio of niclosamide and equivalent volume of nanoparticle with pDNA) for 1h before loading on 1% agarose gel. Gel was run for 30 min on 120 mV in TAE buffer. Untreated pDNA was run as control for the experiment. After running the gel, it was stained in ethidium bromide (20 mg/100 ml) for 10 min and washed for 5 min to remove excess of EB staining. Gel was imaged under UV light.[5]

**Human transformed cancer cell culture**

MD-MB231 cells (ER(-)Breast cancer cells), MCF-7 cells (ER(+)Breast cancer cells) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) supplemented with 10%

fetal bovine serum (FBS) while C32 (human melanoma) was cultured in 10% FBS containing Eagle's minimal essential medium (EMEM) in T25 culture flasks and were incubated at 37 °C in a 99% humidified atmosphere containing 5% $CO_2$. Cells were regularly trypsinized with 0.1% trypsin (EDTA 0.02%, dextrose 0.05%, and trypsin 0.1%) in DPBS (pH 7.4). Non-synchronized cells were used for all the experiments.[1]

**MTT Asssay**

The % cell viability of various cells treated with Niclocelle and nanocelle formulations were investigated for 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay in presence of 10% FBS in antibiotic free media. Experiment was performed in 96 well plates growing 7,000 cells per well 24 h before treatments. Experiments were performed for various concentrations of niclosamide in Niclocelle, PostNiclocelle or free form ranging from 0.5 to 20 μM while Nanocelle was used as negative control. Cells were incubated for two different time points 48 and 72h before performing the MTT assay. After incubation period cells were treated with MTT as 20 ml (5 mg/mL) per well and further incubated for 4 h. At the end of the incubation entire medium was removed from wells and 200 μL DMSO was added to dissolve blue colored formazan crystals. The %-cell viability was obtained from plate reader and was calculated using the formula % Viability = {[$A_{630}$(treated cells)- (background)]/[$A_{630}$(untreated cells)-background]}x100.[1]

**Cell Imaging and Apoptosis Assay**

To establish the apoptotic cell population in cells treated with Niclocelle and nanocelle, flow assisted cell sorting (FACS) analysis on propidium iodide (PI) stained treated and untreated cells were performed. Cells (0.3 x $10^6$ per well) were plated in 6 well plates and grown till it achieved

~80 % confluence. At the end of ~24h incubation, cells were washed with plain DMEM and treated with 20 µM of niclosamide in free or Niclocelle form. At the end of 72h time point, cells were imaged for morphology determination. Cell morphology was monitored by bright field imaging. Cells were trypsinized and collected in 100 µl of reconstituted medium (DMEM containing 10% FBS) and fixed with chilled EtOH 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 triplicated and pooled down before acquiring the FACS data.

**Annexin-V Assay**

To further establish the distinguished early apoptotic and late apoptotic cell population in cells treated with Niclocelle and Nanocelle, FACS analysis of Annexin-V and propidium iodide (PI) dual stained cells were performed. Cells (0.3 x $10^6$ per well) were plated in 6 well plates and grown till it achieved ~80 % confluence. At the end of ~24h incubation, cells were washed with plain DMEM and treated with 20 µM of niclosamide in free or Niclocelle form. At the end of 72h time point, cells were trypsinized and collected in 1 mL of reconstituted medium (DMEM containing 10% FBS) and washed with DPBS at least two times and incubated with PI (2 µg/mL) and Annexin-V (1 µg/mL) for 30 min before scanning on FACS machine.[5]

**Reference**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