Supporting Information

Combinatorial Therapy for Triple Negative Breast Cancer using HyperStar Polymer-based Nanoparticles

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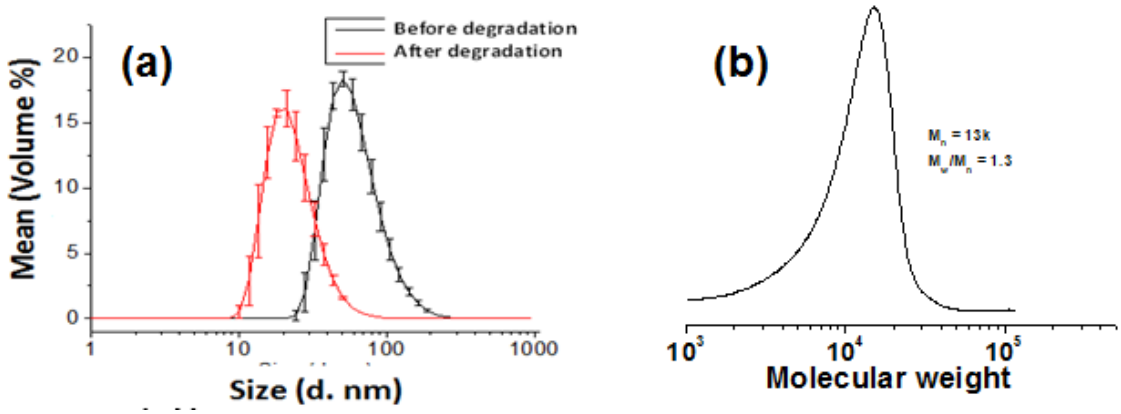

Figure S1. (a) pH dependent degradation of HSPs at pH 3 (red) and change in hydrodynamic diameter of HSP (black) and (b) DMF SEC of degraded product from HSP after dissolving 0.5 mg of A-BIEM-DMAEMA HPS in 1 mL pH 3 sodium citrate buffer for 2 hours.

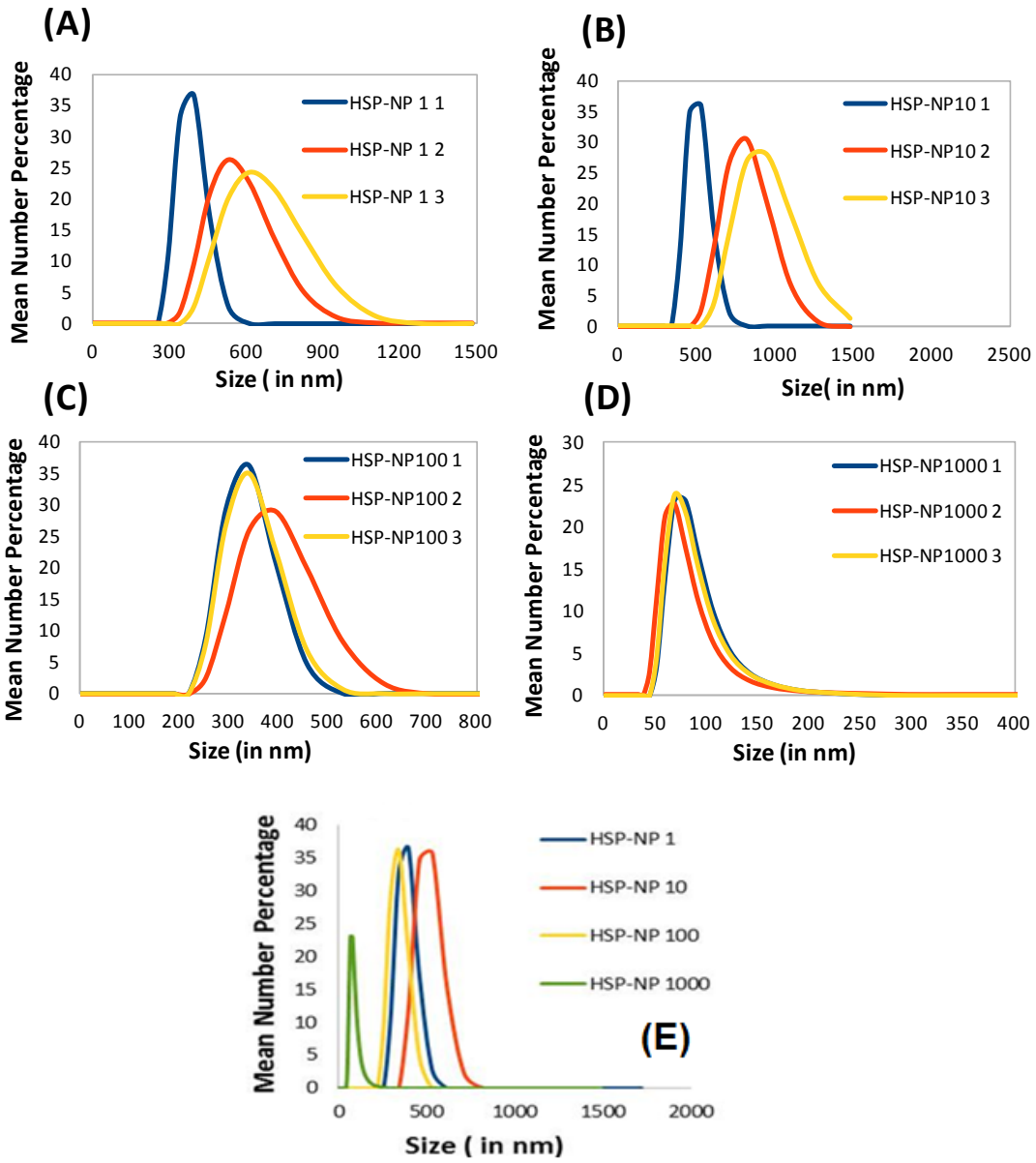

Figure S2. (A-D) Hydrodynamic diameters of HSP NPs prepared by solvent evaporation method at different concentrations of HSP ranging from 1 to 1000 nM with three independent measurements. (E) Summarized hydrodynamic diameter of HSP-NPs at various concentrations of HSPs ranging from 1-1000 nM.

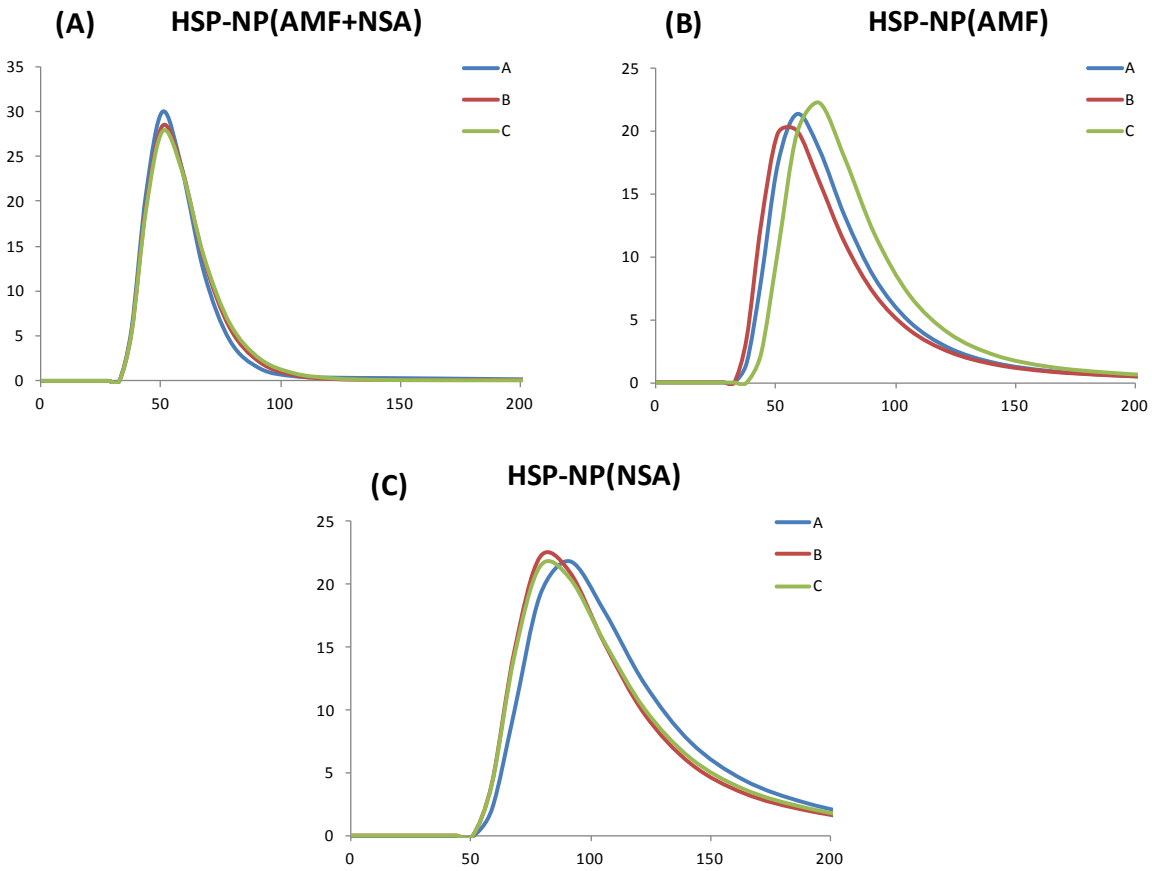

Figure S3. Hydrodynamic diameters of HSP-NPs loaded with drug molecules AMF, NSA and their combinations using solvent evaporation methods with three independent measurements.

Table 1. IC50 of formulations AMF, NSA, AMF+NSA, nano-cocktail and HSP-NP in BT549, MDA-MB231, MCF-7 and SKBR-3 cells.

		IC50 (µM)			
Cells	HSP-NP	AMF	NSA	AMF+NSA	HSP-NP (AMF+NSA)
BT549	-	20±02	30±03	10±02	5±1
MDA-MB231	-	80±10	7±2	5±0.5	1±0.5
MCF-7	-	>100	30±05	40±05	30±05
SKBR-3	-	15±02	15±01	20±05	20±05

[a]Equal molar concentration of AMF and NSA in the feed to prepare the combination AMF+NSA and nano-cocktail HSP-NP (AMF+NSA) formulations.

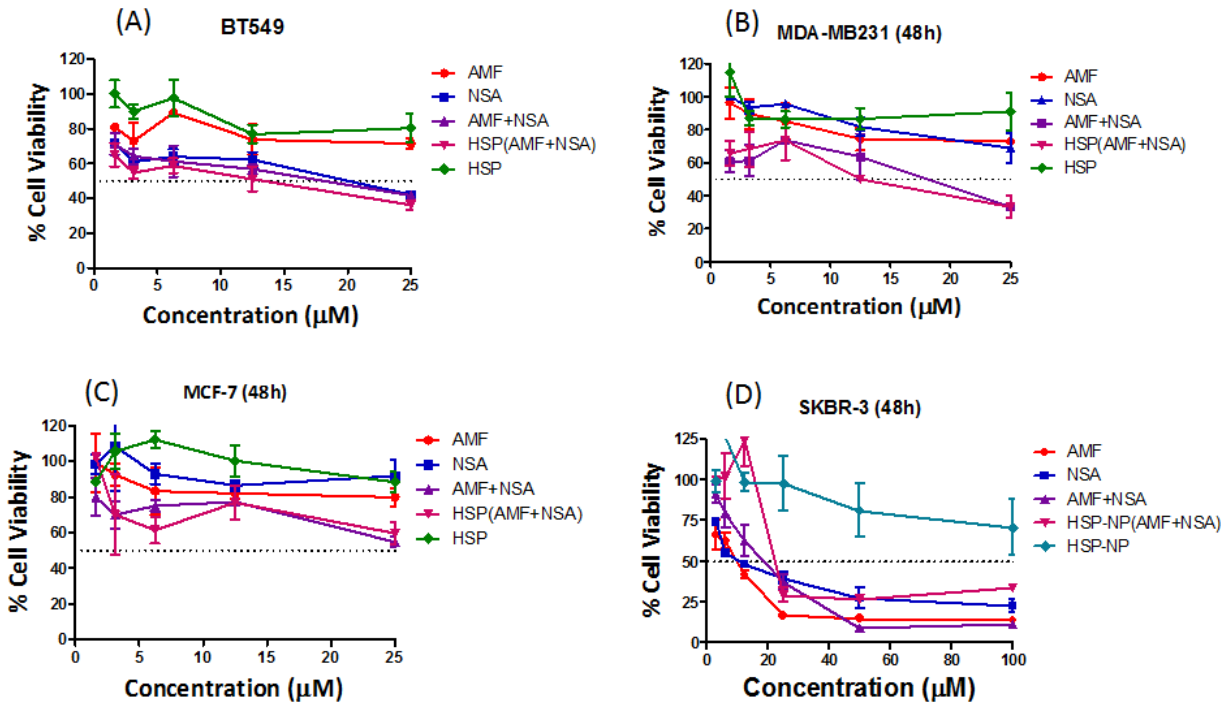

Figure S4. MTT assay in TNBCs, BT549, MDA-MB231, ER(+) MCF-7 and SKBR-3 cells after incubation with DMSO solution of AMF, NSA, AMF+NSA, HSP-NP(AMF+NSA) and HSP-NP at concentrations ranging from 1.875-100 µM for 48h. HSP-NP was used as negative control in all the experiments.

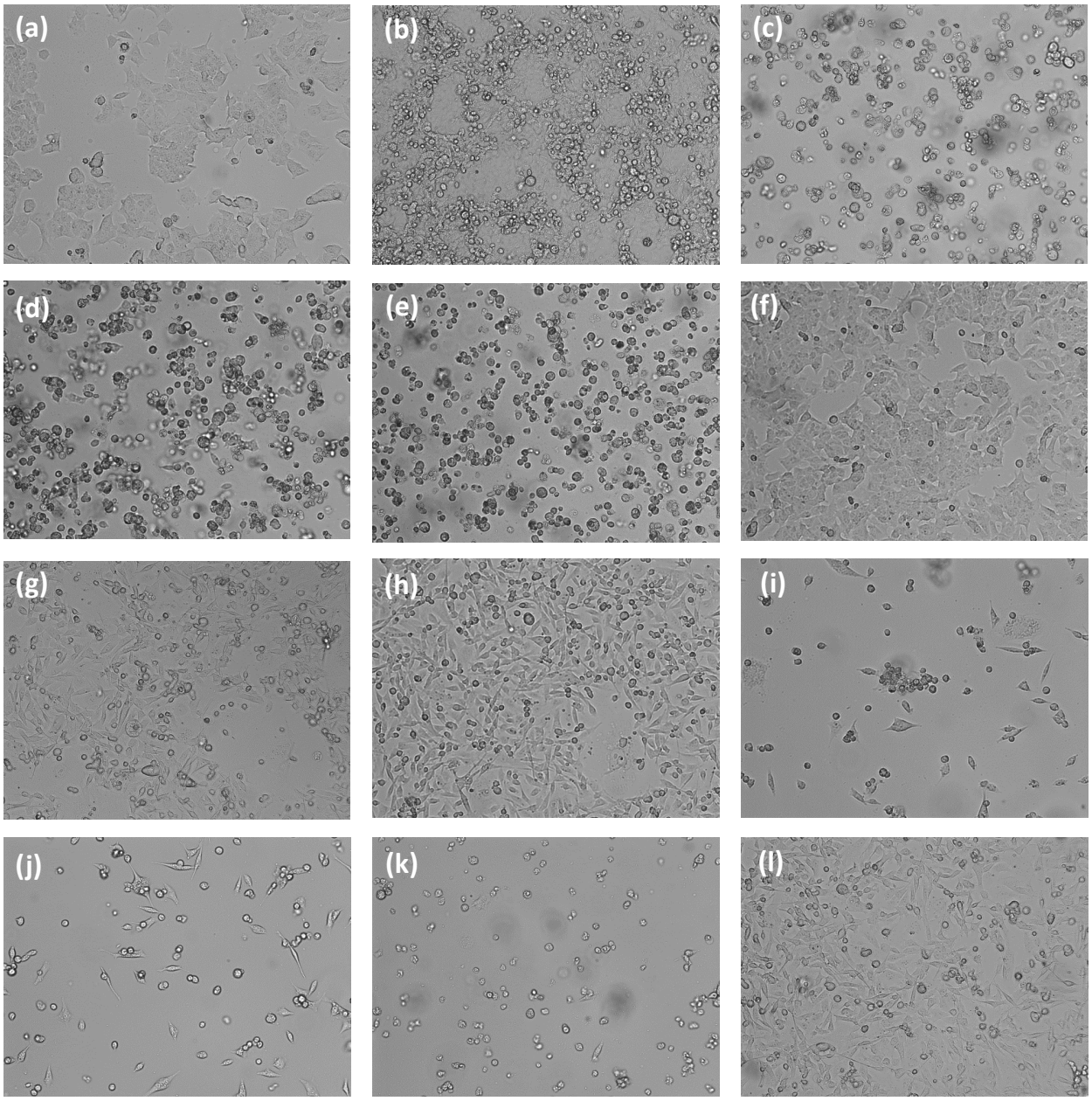

Figure S5. Representative bright field images and morphological variations in cells treated with different formulations. (a-f) MCF-7 and (g-l) MDA-MB231. (a, g) Cells only; (b, h) AMF; (c, i) NSA; (d, j) AMF+NSA; (e, k) HSP-NP (AMF+NSA) and (f, l) HSP-NP.

Figure S6. DNA laddering assay on genomic DNA extracted from the cells treated with different formulations. Lanes represent MCF-7 (1-3, 7, 9 and 11) and NDA-MB231 (4-6, 8, 10 and 12); Lanes 1, 4: cells only; Lanes 2, 5: AMF; Lanes 3, 6: HSP-NP; Lanes 7, 8: NSA; Lanes 9, 10: AMF+NSA and Lanes 11, 12: HSP-NP(AMF+NSA).

Experimental Part

Synthesis of 2-(vinyloxy)ethyl 2-bromo-2-methyl propanoate

A 100 mL round bottom flash was loaded with magnetic stir bar, 2-(vinyloxy)ethanol (10.0 mL), triethyl amine (11.2 mL), and 30 mL dichloromethane (DCM), and then cooled to ~0 ^{o}C in an ice bath. A mixture of 2-bromoisobutyrl bromide (11.9 mL) dissolved in 10 mL DCM was added to the reaction mixture via addition funnel over 30 minutes. The reaction was allowed to warm to room temperature and let react overnight. Once complete, the solid was filtered off and the solvent was removed under reduced pressure. The product was purified with reduced pressure distillation at 70 ^{o}C yielding a clear oil (19.98g, 75.6% yield).

Synthesis of 2-(1-(2-((2-bromo-2-methylpropanoyl)oxy) ethoxy)ethoxy)ethyl methacrylate (inimer 1)

The produced 2-(vinyloxy)ethyl 2-bromo-2-methylpropanoate (3.8 g) was dissolved in 50 mL of DCM along with 2.085 g of 2-hydroxyethyl methacrylate. A catalytic amount of *p*-toluene sulfonic acid was added to start the reaction (30.5 mg). The reaction was allowed to stir for 1 hour and then neutralized with a few drops of TEA. The product was then reduced and purified via column chromatography (2:1, hexanes: ethyl acetate, 3% TEA) yielding a clear oil (5.03g, 85% yield).

Polymerization of inimers using ATRP in microemulsion

A typical polymerization of inimer consisting of $[inimer]_0/[CuBr_2]_0/[dNbpy]_0/[sodium\ ascorbate]_0$ = 70/1/2/0.5 is described. In a disposable test tube, dNbpy and $CuBr_2$ were complexed in 0.5 mL of DCM at 40 ^{o}C for 0.5 hour. Inimer was then added to the complexed

mixture at room temperature before evaporating the DCM. The mixture was added dropwise to a water/Brij98, 12g/1g solution over 30 minutes, ensuring the solution didn't become hazy at any time. During this period, the oil bath temperature was slowly stepped up to 65 ^{o}C before the mixture was initiated with a sodium ascorbate/water solution. The reaction was stopped by exposure to air after 2 hours.

Synthesis of hyperstar polymer (HSP)

The theoretical number of initiating sites per HB1 polymer was assumed to be equal to the average number of inimer units (= $814\times10^3/366 = 2224$). A clean and dry 10 ml Schlenk flask was charged with 40 mg HB1 (0.11 mmol Br), *N,N*-dimethylaminoethyl methacrylate (DMAEMA, 2.0 ml, 12.7 mmol), 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA, 14 μL, 0.051 mmol), acetone (2.5 ml) and anisole 40 μL as internal standard for gas chromatography measurement of DMAEMA conversions. The flask was deoxygenated by five freeze-pump-thaw cycles. During the final cycle, the flask was filled with nitrogen before CuBr (6.5 mg, 0.046 mmol) and $CuBr_2$ (1 mg, 4.5 μmol) were quickly added to the frozen mixture. The flask was sealed with a glass stopper then evacuated and back-filled with nitrogen five times before it was immersed in an oil bath at 40 ^{o}C. Samples were withdrawn periodically for GC measurements of monomer conversions. The reaction was stopped at DMAEMA conversion 27% after 1 hour via exposure to air and dilution with acetone. The solution was filtered through a column filled with neutral alumina to remove the copper complex before the polymer was dialysis against the deionized water overnight and lyophilization.

Degradation of acetal-containing HSP

A stock solution of 0.5 mg of HSP was dissolved in 1 mL pH 3 sodium citrate buffer, the size of the nanoparticle was then monitored by DLS.

Characterization

After filtration through 0.45 μm filter, the polymer samples were separated by SEC with THF or DMF as eluent. The THF SEC was equipped with Polymer Standards Services (PSS) columns (guard, 10^5, 10^3, and 10^2 Å SDV columns) at 35 ^oC and the DMF SEC used Polymer Standards Services (PSS) columns (guard, 10^4, 10^3, and 10^2 Å GRAM 10 columns) at 55 ^oC. Both SECs were set a flow rate = 1.00 mL/min and connected with a differential refractive index (RI) detector (Waters, 2410) using PSS WinGPC 7.5 software. The apparent molecular weights were calculated based on linear poly(methyl methacrylate) (PMMA) standards. The detectors employed to measure the absolute molecular weights of polymers in the THF SEC were a RI detector (Wyatt Technology, Optilab T-rEX) and a multi-angle laser light scattering (MALLS) detector (Wyatt Technology, DAWN HELEOS II) with the light wavelength at 658 nm. Absolute molecular weights were determined using ASTRA software from Wyatt Technology with the dn/dc value of poly(inimer 1) as 0.084 mL/g. The size distribution of the samples was determined by dynamic light scattering (DLS) equipped with a Zetasizer Nano-ZS (He-Ne laser wavelength at 633nm) (Malvern Instruments, Malvern, UK).

Preparation of optimized HSP nanoparticles (HSP-NPs)

A solvent evaporation method was used to prepare nanoparticles from HSPs. A THF solution (10 mM) of HSP was prepared and added dropwise to 1 mL of water heated at 65 °C. Various volumes of THF solution of HSPs (0.1, 1, 10 and 100 μL) were added to prepare HSP-NPs concentration of 1, 10, 100 and 1000 nM, respectively. The solution was left for stirring (1000 rpm) overnight to allow THF evaporation. At the end of the procedure, volume was made up to 1 mL with autoclaved nanopure water (0.2 μM). The suspension was further allowed to stir for 10 min at room temperature. Finally, the suspension was stored at 4 °C overnight before

the particle and size measurement was performed. The nanoparticles were purified by dialysis against nanopure (0.2μm) water using a 10,000 Da MWCO cellulose membrane for prolonged period of time and then passed through a 0.45 μm Acrodisc Syringe filter. The nanoparticles were stored under argon atmosphere typically at 4°C in order to prevent any bacterial growth.

Preparation of drug loaded HSP-NPs

Incorporation of drugs, niclosamide (NSA) and amonafide (AMF), together or individually, was performed by solvent evaporation method as described earlier. A 100 μL THF solution of NSA (0.08 mg/100 μL) and/or AMF (0.07 mg/100 μL) was added dropwise to 1 mL of water heated at 65 °C and co-added the 100 μL of HSP solution in THF (10 mM). The solution was left for stirring (1000 rpm) overnight to allow THF evaporation. At the end of the procedure, volume was made up to 1 mL with autoclaved nanopure water (0.2 μM). The prepared suspensions (HSP-NP(AMF), HSP-NP(NSA) and HSP-NP(AMF+NSA)) were further allowed to stir for 10 min at room temperature. Finally, the suspension was stored at 4 °C overnight before the particle and size measurement was performed. The nanoparticles were purified by dialysis against nanopure (0.2μm) water using a 10,000 Da MWCO cellulose membrane for prolonged period of time and then passed through a 0.45 μm Acrodisc Syringe filter. The nanoparticles were stored under argon atmosphere typically at 4°C in order to prevent any bacterial growth.

Solvent evaporation method is one of the earliest methods used for the preparation of polymeric nanoparticles.[1] Polymer solutions prepared in volatile solvents generate emulsions in aqueous medium. The added pharmacophoric drugs would place themselves in these emulsions based on their hydrophilicity and hydrophobicity. Prepared emulsions would allow nanoparticles diffuse through the continuous phase of the emulsion. The solidified

nanoparticles were obtained after evaporation of the solvent while rate of evaporation significantly decides the final hydrodynamic diameter of the particles.

Dynamic light scattering measurements

Hydrodynamic diameter distribution and distribution averages for HSP-NP, HSP-NP(AMF), HSP-NP(NSA) and HSP-NP(AMF+NSA), in aqueous solutions were determined using a Malvern Zetasizer nano series–Nano ZS90. 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. The measurements for the particles were made at 0, 24, 48, 72, 96, and 120 hours after synthesis to evaluate the stability. All determinations were made in multiples of five consecutive measurements.

Zeta potential measurements

Zeta potential (ζ) values for the HSP-NP, HSP-NP(AMF), HSP-NP(NSA) and HSP-NP(AMF+NSA) were determined with a nano-series Malvern Zetasizer zeta potential analyzer. Measurements were made following dialysis (MWCO 20 kDa dialysis tubing, Spectrum Laboratories, Rancho Dominguez, CA) of nanoparticle suspensions into water. 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 ±5 mV of the mean value given by 20 determinations of 10 data accumulations

Transmission electron microscopy (TEM) measurements

The Transmission electron microscopy (TEM) was performed on HSP-NP and HSP-NP(AMF+NSA) to evaluate their morphologies. Imaging was performed on samples prepared on copper grids that were coated with a formvar plastic and then coated with carbon for stability followed by negative staining with Uranyl acetate.

Human transformed cancer cell culture

We used breast cancer cells of invasive ductal carcinoma trait (MD-MB231 cells (3-ve), MCF-7 cells (ER (+ve)), SKBR-3 (HER-2 (+ve)) and BT549 cells (3-ve)) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma), McCoy's 5A Medium (ATCC® 30-2007™) and RPMI 1640, 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% CO2. 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). Non-synchronized cells were used for all the experiments.

MTT assay

The cell viability with HSP-NP, HSP-NP(AMF), HSP-NP(NSA) and HSP-NP(AMF+NSA) in MD-MB231, BT549, SKBR-3 and MCF-7 cells was investigated by using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT). HSP-NPs were used as negative control for all the experiments. Experiment was performed in 96 well plates (Cellstar®; Germany) growing 8,000 cells per well 24 h before treatments. Experiments were performed for various concentrations of NSA and AMF ranging from 1.625 to 100 μM present in free or nanoparticle forms while same volume of HSP was used as negative controls. Cells were incubated for 48 and 72h before performing the MTT assay. After

incubation period, cells were imaged for investigating growth density and morphology variations. Cells were further treated with MTT as 20 µl (5 mg/mL) per well and further incubated for 4.5 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 percentage 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.

PI staining assay for measurement of apoptotic cell population

At the end of ~24h of incubation, cells were treated with 25 µM of AMF, NSA, AMF+NSA and HSP-NP (AMF+NSA) and incubated for 48h. At the end of incubation period 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.

DNA laddering assay for evaluation of fragmented genomic DNA

At the end of ~24h of incubation, cells were treated with 25 µM of AMF, NSA, AMF+NSA and HSP-NP (AMF+NSA) and incubated for 48h. At the end of incubation period cell morphology was monitored by bright field imaging. 48h post incubation cells were trypsinized and collected in 400 µL of lysis buffer. The genomic 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.

Topo-II Assay

MDA-MB231 (ER(-) breast cancer) cells were seeded at 2,000,000 cells/plate on a 100 mm^2 tissue culture dish. After 24 hours of incubation at 37 °C and 5% CO_2, the cells were treated with a 25 μM concentration of AMF (Amonafide, Sigma Aldrich, St. Louis, MO), NSA (Niclosamide, Sigma Aldrich, St. Louis, MO), and a nanococktail of AMF and NSA for 48 hours. Trypsin-EDTA was added to the culture dishes and pipetted out immediately. The dishes were incubated for 3 minutes at 37°C and 5% CO_2, and the cells were washed and collected in DPBS. The 15 mL tubes were centrifuged at 800 x g for 3 minutes at 4°C, and the supernatant was discarded. The cell pellet was resuspended in 4 mL of cold TEMP buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 4 mM MgCl2, 0.5 mM PMSF) and pipetted to mix. The suspension was centrifuged again at 800 x g for 3 minutes at 4°C. The supernatant was discarded, and 3 mL of cold TEMP buffer was added to each cell pellet. The cell suspension was placed on ice for 10 minutes, then probe sonicated three times (Amp:1, ON: 5 sec, OFF: 30 sec). To pellet the nuclei, the cell suspension was centrifuged at 15,000 x g for 15 minutes at 4°C. After discarding the supernatant, the pellet was resuspended in 1 mL of cold TEMP buffer, and transferred to a 1.5 mL Eppendorf tube, then centrifuged again at 15,000 x g for 15 minutes at 4°C. The supernatant was discarded and 200 μL of cold TEP buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM PMSF) was added. In the same suspension, 200 μL of cold 1M NaCl was also added, and the pellet was resuspended via vortexing and pipetting. The suspension was placed on ice for 30 min before being centrifuged at 100,000 x g for 1 hour at 4°C. The supernatant was collected in a 1.5 mL Eppendorf tube and stored overnight at 4 °C.

To prepare the samples for gel electrophoresis, the Topoisomerase II Assay Kit (TopoGEN, Inc., Port Orange, FL) was used. Sample mixtures were prepared using 1,2,4,8 and 16 μL of the supernatant with 1 μL of DNA (kDNA, decatenated, or catenated), 5 μL of

the 5x Complete Reaction Buffer, and made up to 22 µL by adding autoclaved water in a 1.5 mL Eppendorf tube. The samples were placed in a water bath at 37 °C for 30 minutes, and the reaction was stopped by the addition of 5 µL of 5X Stop Buffer. All samples were loaded onto a 1% agarose gel, and the gel was run for 45 minutes at 100 V. The gel was stained in 3% v/v ethidium bromide solution (10 mg/mL) for 5 min and washed in ethidium bromide solution for 5 min before being imaged under gel doc (Universal Hood III, Bio-Rad, Hercules, CA).

RNA extraction and PCR analysis

RNA was collected from untreated and treated MDA-MB231 cells using an RNeasy Mini Kit (Qiagen) following manufacturers protocols. cDNA was synthesized by incubating 125 ng of purified RNA with 4 uL of gene specific reverse primers (1 uM), and 2 uL of dNTPs (10nM) in total volume of 26 uL at 65 °C for 5 minutes followed by incubation at 4 °C for 5 minutes. 5′GCTTCCTGCAAGAGTCGAAT3′ as forward and 5′ATTGGCTTCTCAA GATACCTG3′as reverse primers were used against STAT-3 and 5'-GAGCGCGGCTACAGCTT-3' Forward, 5'-TCCTTAATGTCACGCACGATTT-3' Reverse for b-actin. At 4 °C, 2 uL of Superscript III Reverse Transcriptase (200 U/uL; Invitrogen), 2 uL DTT (100 mM), 8 uL of 5x buffer, and 2 uL RNase Out (40 U/uL; Invitrogen) were mixed and the reaction incubated for 1 hour at 55 °C followed by incubation for 15 minutes at 70 °C. Quantitative PCR was performed using StepOnePlus RT PCR system (Applied Biosystems). Each qPCR reaction used 2 uL directly from the cDNA synthesis, 1 uL forward primer (8 uM), 1 uL reverse primer (8uM), and 10 uL FastStart Universal SYBR Green Master (Thermo Fisher Scientific) in a total volume of 20 uL. RT-PCR cycle was as follows: 95 °C for 8 min, 35 cycles of (95 °C for 4 sec, 56 °C for 6 sec, 72 °C for 16 sec), and a melt curve of 95 °C for 15 sec, 58 °C for 30 sec, and ramp to 95 °C.

Plausible mechanism of drug release in vitro

As established by pH mediated degradation, experiments were performed to establish the possible pathway of drug release from Nic-Am-HSP-NP at pH 4.0. Samples were incubated at pH 4.0 for 4h before acquiring UV-Vis absorption pattern indicating release of loaded drugs.

Statistical analysis

Statistical significance of differences between control and samples were evaluated using one-way ANOVA using GraphPad Prizm 5.0 with Dunnett or Bonferroni analysis wherever applicable. Results were considered statistically significant when the p value was less than 0.05.

The CI has been analysed using the formula:

CI = (Cax/ICxa)+(Cbx/ICxb)

where Cax and Cbx are the concentrations of drug A and B used in combination to achieve x % drug effect. ICxa and ICxb are the concentrations for single agents to achieve the same effect. A CI of less than, equal to, or more than 1 indicates synergic, additive or antagonistic effect, respectively.[2]

CI (BT549) = 5/20+5/30 = 0.41

References.

[1] B. V. N. Nagavarma, H. K. S. Yadav, A. Ayaz, L. S. Vasudha, H. G. Shivakumar, Different techniques for preparation of polymeric nanoparticles-A review, *Asian J Pharm Clin. Res.* **2012***, 5,*16.

[2] F. A. Piazza, M. Ruzzene, C. Gurrieri, B. Montini, L. Bonanni, G. Chioetto, G. D. Maira, F. Barbon, A. Cabrelle, R. Zambello, F. Adami, L. Trentin, L. A. Pinna, G. Semenzato,

Multiple myeloma cell survival relies on high activity of protein kinase CK2. *Blood*. **2006**, *108*, 1698.