This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal’s standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.

This article can be cited before page numbers have been issued, to do this please use: A. K. Jangid, D. P. Kulhari, P. Jain, S. V. K. Rompicharla, S. Ramesan and H. Kulhari, Mater. Adv., 2020, DOI: 10.1039/D0MA00189A.
1. Introduction

In past two decades, various drug delivery systems have been developed to improve the therapeutic efficacy and site-specific delivery of anticancer drugs. Most of these systems are based on nanoscience and nanotechnology. Initially developed nanoscale drug delivery systems were based on passive targeting. In this approach, drug loaded nanocarriers i.e. nanomedicines are preferentially accumulate in the tumor tissue due to the enhanced permeability and retention effect resulting from leaky vasculature and impaired lymphatics drainage in rapid-growing tumors. Later on, actively targeting nanomedicines were designed to improve the selectivity of nanomedicines towards cancer cells. In this approach, a targeting ligand is conjugated or coated on the surface of the drug loaded nanoparticles which enhance the binding and uptake of nanoparticles by cancer cells through receptor-ligand interaction. Thus, this approach is mostly dependent on the over expression of receptors on cancer cells in comparison to healthy cells. Although, several preclinical studies have demonstrated the applicability of this approach but the efficacy of targeted nanoparticles is restricted by stochastic nature of ligand–receptor interactions, tumor heterogeneity, hypoxia, difficulty in control over the release of drug, and endosomal escape.

Stimuli-responsive nanoscale delivery system (Sr-NDS) is a powerful strategy to design advanced therapeutic delivery system for the treatment of different types of cancer. Sr-NDS respond in dynamic way to a particular stimulus by recognizing its microenvironment which may be a change in pH, temperature, biochemical levels, presence of a particular enzyme, magnetic field etc. For designing a successful Sr-NDS, a biomaterial is required which not only have the physicochemical properties to respond the stimulus but is also biodegradable and biocompatible. Further, sensitivity towards the stimulus is very important for the selective delivery of the drugs, especially in case of the anticancer nanomedicines.

The aim of this research work was to design a Sr-NDS for improving the delivery of cabazitaxel (CTX) a chemotherapeutic antineoplastic agent approved by FDA in 2010 for the treatment of hormone-refractory prostate cancer. CTX is second-generation taxane which is specifically designed for hormone-refractory prostate cancer treatment. CTX was initially formulated in polysorbate 80 and ethanol (13%), known as JEVTVANA® that have been widely used formulation in clinical settings. However, clinical use of CTX is associated with serious side effects such as hypersensitivity reactions, neurotoxicity, bone marrow suppression, fluid retention, diarrhoea, nausea, vomiting, numbness, and hair loss etc. due to the non-specific distribution to healthy cells. Alternatively, nanoformulations such as micelles, liposomes, albumin nanoparticles and mPEG-PLA-CTX conjugated nanoparticles have been developed to eliminate the drawbacks of CTX and to deliver to the cancer cells.
formulations have been proved successfully for the enhancement in the therapeutic efficacy of CTX but these formulations still showing drawbacks like low drug loading capacity, instability in body fluid, and burst drug release.

To overcome these issues, we have developed self-assembled, pH-sensitive, water-soluble, and stable nanoformulation of CTX through its direct conjugation with a biocompatible polymer. For designing of CTX-based formulation, pluronic F68 (PF68) was selected as polymeric material because it is highly biocompatible, biodegradable, has well known safety profile, can self-assemble in aqueous media and moreover, and it has p-glycoprotein inhibition property that also helps to overcome the drug resistance problem for cancer treatment. For the conjugation of CTX to PF68, two pH-sensitive linkers i.e. succinic anhydride and cis-aconitic anhydride were selected to covert the designed formulation into Sr-NDS. The slightly acidic environment of the tumor tissues (pH 6.5) and the significant differences in the pH of endosomes (pH 5–6) and lysosomes (pH 4–5) from physiological or blood pH (pH 7.4) and provide opportunity to design the pH-responsive formulations for cancer treatment. Among different types of Sr-NDS, pH-sensitive Sr-NDS is preferred system because of its well-established protocols, use of low-cost chemicals, simplicity, and non-requirement of specific equipment. To exploit this phenomenon, various pH-sensitive formulations have been developed previously. The conjugation of polymer and drug conjugate to prepare pH-sensitive prodrug nanomicelles have widespread concern.

Recently, various polymer-drug conjugates have been developed through pH-sensitive linkers like succinoyl, acetal, cis-acetinyl, hydrazone, thiols, schiff base, imine, borate and developed through pH-sensitive linkers like succinoyl, acetal, cis-acetinyl, hydrazone, thiols, schiff base, imine, borate and oximes. However, the acid-labile chemical bonds have received more attention for development of pH-sensitive drug delivery systems to cancer therapy. The acid-labile linker containing polymer-drug conjugate can only release the drug at acidic environment into cancer cells. Hence, in this present study, a polymer-drug conjugated prodrug with acid-labile bond was designed to remain stable in blood pH (7.4), and quickly degrade in mild acidic condition of cancer cells.

2. Experimental section

2.1 Materials

Cabazitaxel (CTX), 4-dimethylaminopyridine (DMAP), cis-aconitic anhydride (CAA), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), hydroxybenzo triazole (HOBt), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), calcein-AM, annexin V-FITC kit, bis benzimide H 33342 trihydrochloride (Hoechst 33342), JC-1, 2,7'-Dichlorofluorescin diacetate (DCFDA), fetal bovine serum (FBS), glutamine, penicillin and streptomycin were purchased from Sigma Aldrich (St. Louis, MO, USA). Succinic anhydride (SA), triethylamine (TEA), diethyl ether and dichloromethane (DCM) were purchased from Rankem (Mumbai, India). Pluronic F68 (F68) was received as a gift sample from BASF (New Jersey, USA).

2.2 Synthesis of pluronic F68 and cabazitaxel conjugates via pH sensitive linkers

The conjugation of CTX to pluronic F68 via succinoyl and cis-aconityl linkages (abbreviated as F68-SA-CTX and F68-CAA-CTX, respectively) was carried out by the two steps synthesis reaction. Firstly, the hydroxyl group of pluronic F68 (F68) was converted into succinoyl and cis-aconityl groups using succinic anhydride and cis-aconitic anhydride, respectively. In second step succinoyl F68 or cis-aconityl F68 (1.0 eq), EDC (1.2 eq) and HOBt (1.2 eq) were dissolved in anhydrous dichloromethane (DCM) and stirred magnetically for 12 h under N\textsubscript{2} condition. After the activation of carboxyl group, CTX (1.0 eq) and DMAP (1.0 eq) (solubilized in DCM) were added and the reaction was maintained at room temperature for 24 h under N\textsubscript{2} condition. After that, the reaction mixture was transferred in a separating funnel and brine solution (0.9% NaCl) was added. The DCM layer was separated and dried using anhydrous Na\textsubscript{2}SO\textsubscript{4}. The DCM was evaporated by the Rotavapour (IKA RV10 Digital V), vacuum dried the obtained product and used for further studies.

2.3 Characterizations of F68-SA-CTX and F68-CAA-CTX conjugates

The proton NMR spectra of synthesized conjugates were recorded using Bruker 500-MHz Ultra shield plus NMR instrument after dissolving the samples in CDCl\textsubscript{3}. For the FTIR analysis, the synthesized F68-SA-CTX and F68-CAA-CTX were pelletized with Kbr and scanned for % transmittance using Perkin Elmer Spectrum 65 series instrument from 4000 to 4000 cm\textsuperscript{-1}.

2.4 Determination of critical micelles concentration of F68-CTX conjugates

The critical micelle concentrations of F68-SA-CTX and F68-CAA-CTX conjugates were determined by our previously validated and reported method. A volume of 25 µL of pyrene solution (6x10\textsuperscript{-7} M) was incubated with various concentrations (50 to 600 µg/mL) of F68-CTX conjugates at room temperature for 3 h. The samples were excited at wavelength at 339 nm and emission fluorescence intensities were measured at 383 and 373 nm using a microplate reader (Synergy H1 Hybrid Reader, Biotek; Winooski, VT, USA). The intensity ratio I\textsubscript{383}/I\textsubscript{373} verses Log C (µg/mL) were plotted for the determination of CMC values.

2.5 Preparation of nanomicelles of F68-CTX conjugates

Briefly, 10 mg of F68-SA-CTX or F68-CAA-CTX conjugate was solubilized in 1 mL of acetone and added to the 5 mL of water. The prepared dispersion was stirred magnetically at 1000 rpm for 3 h to evaporate acetone. The resultant micelles were filtered through 0.2 µm syringe filter and used for further experiments. For storage, nanomicelles were lyophilized and kept in refrigerator until further use. The prepared F68-SA-CTX and F68-CAA-CTX conjugate nanomicelles were termed as CNM-1 and CNM-2, respectively. The percentage drug loading (% DL) of CTX in the CNM-1 and CNM-2 was measured by UV-visible spectrophotometer. The maximum absorbance of CTX was determined at 230 nm. The % DL of CTX was calculated by the following formula:

\[
% \text{DL} = \frac{\text{Amount of CTX in nanomicelles}}{\text{Weight of F68} – \text{CTX conjugate}} \times 100
\]

2.6 Characterization of F68-CTX conjugated nanomicelles

2.6.1 Particle size and zeta potential analysis

Particle size (nm), polydispersity index (PDI) and zeta potential (mV) of CNM-1 and CNM-2 were measured using a Zetasizer Nano ZS (Malvern Instruments, UK) at room temperature.

2.6.2 Transmittance electron microscopic analysis

The shape of the prepared CNM-1 and CNM-2 were observed by transmittance electron microscope (TEM). For this, a drop of nanomicelles was placed on the carbon coated copper grid, vacuum dried and used for TEM analysis.

2.6.3 X-ray diffraction (XRD) analysis

The powder XRD patterns of the pure CTX, CNM-1 and CNM-2 were obtained using an X-ray diffractometer instrument (D8 Advance, Bruker, Germany) equipped with a Cu-Kα X-ray radiation
2.6.4 Differential scanning calorimetry (DSC) analysis
The DSC analysis of pure CTX, CNM-1 and CNM-2 were analysed on DSC-4000 (PerkinElmer) instrument. The whole process was carried out at temperature range from 30 to 350 °C with heating rate 10 °C/min and under N₂ environment.

2.7 pH-responsive CTX release
The in-vitro CTX release study was performed in two different media i.e. phosphate buffer saline (PBS, pH 7.4) and sodium acetate buffer (SAB, pH 5.0) at 37 °C. Individually CNM-1 and CNM-2 (equivalent of 2 mg of CTX) were transferred into dialysis bag (MWCO 1000 Da) and then the CNM-1 or CNM-2 containing dialysis bag was placed in 100 mL of release media (PBS pH 7.4 or SAB pH 5.0). At predetermined time intervals, 2 mL of media was taken out and fresh 2 mL of media was added to maintain sink condition. The withdrawn samples were analysed for % drug content using UV-visible spectrophotometer.

2.8 Stability study of the nanomicelles
The colloidal stability of CNM-1 and CNM-2 was determined by measuring particle size (nm) and polydispersity index (PDI) using a Zetasizer Nano ZS 90 (Malvern Instruments, UK) at different time intervals. After 24 h, samples were also analysed for the change in shape of the CNM-1 and CNM-2 by the TEM analysis.

2.9 Hemolytic toxicity assay
Hemolytic toxicity of the CNM-1 and CNM-2 was evaluated according to our previously reported method. Briefly, fresh human blood was collected in heparinized tubes and centrifuged at 4000 rpm for 10 min. The supernatant/plasma was collected, and erythrocyte suspension was washed twice with normal saline. The erythrocytes were resuspended in normal saline to make 2% v/v suspension. Then, 2 mL of media was added to maintain sink condition. The withdrawn samples were analysed for % drug content using UV-visible spectrophotometer.

2.10 Cell culture
PC-3 human prostate cancer cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA) and was maintained in Roswell Park Memorial Institute RPMI 1640 medium (Invitrogen, NY) supplemented with 10% Fetal Bovine serum (FBS), glutamine (2 nmol/L), penicillin (100 ng/mL) and streptomycin (100 ng/mL) at a constant temperature of 37 °C and 5% CO₂. The cells were passaged after achieving 70% confluency and the medium was replaced on alternative days. The cells were trypsinised using 0.05% trypsin-EDTA and used between 3-10 passages for experiments.

2.11 Determination of 2D anti-proliferative activity
The 2D cytotoxicity study of the pure CTX, CNM-1 and CNM-2 was carried out against PC3 cells with the aid of MTT assay. About 5×10^4 cells/mL were seeded in 96-well flat bottom plates and allowed to adhere for 24 h before treatments. At 72 h post treatment, 100 μL of 0.5 mg/mL MTT reagent in media was added into each well. The solution was further incubated at 37 °C for 4 h before solubilising the formed formazan crystals in 100 μL of DMSO. The absorbance of the obtained purple/colourless solution was read immediately using a microplate reader (Spectramax) at 570 nm. The percent growth inhibition was calculated as follows: % Growth inhibition = (Mean absorbance of drug treated cells / Mean absorbance untreated cells) × 100. The IC₅₀ values were calculated using the Probit software.

2.12 3D cell viability assay
PC3 cells were seeded at a seeding density of 3×10^4 cells/mL in Corning® Costar®Ultra-Low attachment 24-well plates. The cells were incubated for 3 days to form a complete spheroid structure in complete RPMI media. The pure CTX, CNM-1 and CNM-2 were added at IC₅₀ concentrations and was incubated for 72 h prior to the addition of Calcein AM and propidium iodide (PI) to stain the live and dead cells respectively. Images were taken using the ZOE™ Fluorescent Cell Imager (Bio-Rad Laboratories Inc., Hercules, CA, USA) in green and red channels. Quantitative measurements were carried out using cell titre glow assay. For this, the cells were seeded in Corning 96-well black, spheroid microplates and left to adhere for 3 days similar to the aforementioned live/ dead assay. A volume of 100 μL of CellTiter-Glo® reagent was added to each well containing 100 μL of CTX, CNM-1 and CNM-2 in complete medium. The plates were shaken for 2 min on a shaker to induce cell lysis incubated at room temperature for 10 min before recording the luminescence of the samples.

2.13 Cell cycle analysis
PC3 cells (8×10⁴ cells/well) were seeded in a 12-well plates and were further incubated overnight for attachment. Cells were treated with CTX, CNM-1 and CNM-2 equivalent to IC₅₀ concentration after 24 h. The vehicle treated wells served as the controls. After 24 h of treatment, each sample containing both the floating and the adherent cells was collected and was washed with 150 mM PBS pH 7.4. The obtained pellet was further resuspended in 1 mL of PBS, and added to 9 mL of 70% ethanol with simultaneous vortexing at high speed. The cell suspension was stored at 4 °C for 30 min before the removal of ethanol by centrifugation. The obtained pellet was loosened by gentle tapping and 5 mL of PBS was added to the centrifuge tubes. The tubes were left undisturbed for 15 min to allow sufficient rehydration before centrifugation of the samples at 1000 rpm for 5 min. After 15 min of incubation, 1 mL of propidium iodide staining buffer (PI (200 mg), 0.1% (v/v) Triton X-100, 2 mg DNAse-free RNAse A (Sigma) in 10 mL of PBS) was added to each sample. The stained samples were further stained with Hoechst 33242 (5 μg/mL) at room temperature for a period of 15 min before washing off the excess dye. The stained samples were further incubated overnight for attachment. Cells were treated with CTX, CNM-1 and CNM-2 equivalent to IC₅₀ concentration and incubated for 24 h. The vehicle treated wells served as the controls. After 24 h of treatment, each sample containing both the floating and the adherent cells was collected and was washed with 150 mM PBS pH 7.4. The obtained samples were further stained with Hoechst 33242 (5 μg/mL) at room temperature for 15 min before washing off the excess dye. The stained samples were further incubated overnight for attachment. The stained samples were further stained with Hoechst 33242 (5 μg/mL) at room temperature for 24 h before treatments. At 72 h post treatment, 100 μL of 0.5 mg/mL MTT reagent in media was added into each well. The solution was further incubated at 37 °C for 4 h before solubilising the formed formazan crystals in 100 μL of DMSO. The absorbance of the obtained purple/colourless solution was read immediately using a microplate reader (Spectramax) at 570 nm. The percent growth inhibition was calculated as follows: % Growth inhibition = (Mean absorbance of drug treated cells / Mean absorbance untreated cells) × 100. The IC₅₀ values were calculated using the Probit software.

2.14 Hoechst staining
The cells were seeded at a density of 2×10⁴ /well in a 24-well cell culture plates and incubated for 24 h to render good cell attachment. The culture media was then replaced with media containing CTX, CNM-1 and CNM-2 at IC₅₀ concentrations and incubated for 24 h. The treated cells were fixed with 4% paraformaldehyde which were further stained with Hoechst 33242 (5 μg/mL) for a period of 15 min at room temperature. The stained cells were washed with 150 mM PBS to remove excess, unbound dye prior to imaging under fluorescence microscopy (filters, excitation 350 nm and emissions...
460 nm) to detect apoptotic cells by observing the intensity of the blue coloured nuclei.

2.15 Apoptosis assay by Annexin V-PI staining

To quantify the extent of apoptosis induced by the CTX, CNM-1 and CNM-2, flow cytometric analysis was carried out using Annexin V/propidium iodide (PI) Apoptosis detection kit. The PC3 cells were seeded at 8×10^4/well in a 12-well plate and left overnight for cell adherence. The cells were treated with the CTX, CNM-1 and CNM-2 at IC_{50} concentrations for 24 h before washing the cells with 150 mM PBS. The washed cells were trypsinised and resuspended in 5 µL Annexin-V/fluorescein isothiocyanate (FITC) and 10 µL PI. The cell suspension was incubated in dark for 15 min at room temperature before being analysed for red (FL-2) and green channels (FL-1) using a BD Accuri™ C6 Flow Cytometer (BD Biosciences, San Jose, CA, USA).

2.16 Measurement of reactive oxygen species (ROS) levels

The ROS measurement was accompanied by the addition of DCFDA containing media to the treated cells as mentioned above for other assays. The ROS assay was carried out in a 24 well plate with 2×10^4 cells /well. After the treatment, the media was replaced with culture media containing 10 µM carboxy-DCFDA which was further incubated for 30 min at room temperature. All the incubation was carried out in dark to protect the light sensitive DCFDA compound. The cells were washed with PBS and fluorescence images were captured using a microscope (ZOE™ Fluorescent Cell Imager, Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.17 Measurement of mitochondrial membrane potential (MMP)

PC3 cells were seeded (2×10^4 /well) in a 24-well plate and allowed to adhere overnight. Cells were treated with the CTX, CNM-1 and CNM-2 at IC_{50} concentrations. After 24 h treatment, cells were washed with 150 mM PBS (pH 7.4). A stock solution of 2 mg/mL JC-1 dye was prepared and stored at ~20 °C prior to the staining procedure. To measure the MMP, the stock JC-1 aliquot was reconstituted to a final concentration of 1 µg/mL in 150 mM PBS, with the addition of 0.5 mL of the prepared JC-1 solution to each well. The cells were incubated at 37 °C for 20 min. The structural influence of the drugs on the mitochondrial membrane was inferred from its potential state by examining the relative fluorescence between its green monomeric (depolarised) form at 585 nm via excitation at 514 nm with that of its red aggregated (hyperpolarised) form at 590 nm through excitation at 529 nm, using a microscope (ZOE™ Fluorescent Cell Imager, Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.18 Statistical analysis

Data are expressed as the mean ± standard deviation. Significant differences were calculated by ANOVA analysis and p values < 0.05 were considered statistically significant.

3. Results and discussion

3.1 Charaterizations of PF-CTX conjugate

The conjugation of CTX to F68 was carried by two steps chemical reaction (Figure S1). In first step, the –OH terminated pluronic F68 was converted into –COOH group containing F68 by conjugating with succinic anhydride (SA) and cis-aconityl anhydride (CAA). These succinoyl F68 and cis-aconityl F68 were further conjugated to CTX through esterification reaction. The finally synthesized conjugates (F68-SA-CTX and F68-CAA-CTX) were characterized by the 1H-NMR (Figure 1) and FTIR analysis (Figure S2). The succinoyl –CH2 peaks at δ 2.65–2.67 ppm, and peaks of cis-aconityl F68 at δ 6.71 and 6.36 ppm (alkene) and δ 2.08 ppm (–CH2) confirmed the successfully formation of succinoyl F68 and cis-aconityl F68, respectively. In case of F68-SA-CTX conjugate, the phenyl peaks of CTX (δ 7.06 – 7.61 ppm) and characteristic peaks of F68 (δ 3.65 ppm –CH2 of PEO of F68, δ 2.78 ppm (–CH3 of PPO of F68), δ 2.18–2.66 (–CH2–CH=CH–O–) of PPO of F68 and δ 0.88 – 1.47 (CH3–O– of PPO of F68) confirmed the synthesis of F68-CTX conjugate. Similarly, the phenyl peaks of CTX (δ 7.33 – 7.59 ppm) and characteristic peaks of F68 (δ 3.65 ppm –CH2 of PEO of F68, δ 2.96 ppm (–CH3 of PPO of F68), δ 2.65–2.82 (–CH2–CH=CH–O–) of PPO of F68 and δ 0.84 – 1.88 (CH3–O– of PPO of F68) were clearly observed in F68-CAA-CTX conjugate (Figure 1).

Figure 1. 1H-NMR spectra of (1) pure pluronic F68, (2) succinoyl F68, (3) cis-aconityl F68, (4) pure cabazitaxel, (5) pluronic F68-cabazitaxel conjugate via succinoyl linkage (F68-SA-CTX), and (6) pluronic F68-cabazitaxel conjugate via cis-aconityl linkage (F68-CAA-CTX).

The synthesis of F68-CTX conjugates was further confirmed by the FTIR analysis (Figure S2). In the FTIR spectrum of F68-SA-CTX, the peaks were observed at 1713 and 1648 cm⁻¹ (C=O stretching)16, 1563, 1464 and 1351 cm⁻¹ (C=C stretching, –CH3 bending and –C=N stretching of CTX) confirmed the successfully conjugation of F68-SA-CTX. Similarly, in the FTIR spectrum of F68-CAA-CTX, the peaks were observed at 1723 and 1643 cm⁻¹ (C=O stretching), 1561, 1466 and 1346 cm⁻¹ (C=C stretching, –CH3 bending and –C=N stretching of CTX) were clearly observed in FTIR spectrum of F68-CAA-CTX conjugate. Therefore, the presence of both CTX and F68 FTIR peaks in F68-SA-CTX and F68-CAA-CTX conjugates confirmed the successfully conjugation.
3.2 Determination of critical micelle concentration

CMC values of a micelle system is an important parameter which indicates its stability after administration into the body. A lower CMC value is desirable to form stable micelles. To consider the self-assembling behaviour of F68-CTX conjugates, the CMC was determined by the fluorescence pyrene method. The emission intensity ratio ($I_{373}/I_{383}$) drastically changed from 200 µg/mL concentration (Figure S3) which confirmed the CMC of the F68-CTX conjugates. The CMC value for pluronic F68 is reported to be 4.008 mg/mL.26 Thus, the CMC values of F68-SA-CTX and F68-CAA-CTX conjugates were 20 times lesser than the CMC of F68. The CMC of both conjugates was observed same which confirmed that there was no significant role of linker in micelles formation. The observed lower CMC value of F68–CTX conjugates indicated their more stability than the micelles formed by native pluronic F68.40,41

3.3 Physicochemical characterizations of nanomicelles

The size of the prepared nanomicelles was determined by the DLS and TEM analysis. The particle size, polydispersity index and zeta potential of the prepared nanomicelles were found to be 95.7 ± 3.5 nm, 0.322 ± 0.015 and −13.6 ± 1.3 mV for CNM-1, and 94.8 ± 5.1 nm, 0.315 ± 0.008 PDI and −15.9 ± 1.1 mV for CNM-2, respectively. The observed results suggested the formation of monodispersed nanomicelles of both synthesized conjugates in water after self-assembly. The resulting CNM-1 and CNM-2 showed % DL were 6.32 ±0.22% and 6.21 ±0.30%, respectively. Figures 2a-4d showed the TEM images and SAED patterns of nanomicelles which were spherical in shape with 58.9 ± 7.7 nm and 42.5 ± 3.7 nm for CNM-1 and CNM-2, respectively. The SAED patterns of CNM-1 and CNM-2 did not show any diffraction spots and therefore confirmed the amorphous nature of nanomicelles. To determine the physical state of the CNM-1 and CNM-2, DSC and PXRD analysis were performed. As shown in Figure 2e, the pure CTX showed the characteristic endothermic peak at 178 °C corresponding to its melting point. This typical peak of CTX was not observed in the CNM-1 and CNM-2 which clearly suggested that CTX was molecularly dispersed in the core of the nanomicelles. Figure 2f represented the XRD patterns of pure CTX and CNM-1 and CNM-2. The XRD pattern of CTX clearly shows the number of sharp peaks at 2θ angle 7.0°, 7.8°, 8.9°, 10.1°, 12.3°, 12.6°, 14.1°, 15.5°, 17.5°, 19.2°, 20.9°, 21.8°, 22.9°, 23°, 27.2° and 32.1° indicating the crystalline nature of pure hydrophobic CTX. However, with CNM-1 and CNM-2, no such sharp peaks of CTX were observed. The CNM-1 and CNM-2 showed only two sharp peaks at 2θ angle 18.8° and 23.1° which were obtained due to the presence of the F68. This data further confirmed that the drug was in amorphous phase in nanomicelles.

3.4 Stimuli responsive stability of nanomicelles

For the clinical application of the therapeutic nanomicelles, high physicochemical stability is required to achieve long term blood circulation and accumulation in tumor tissues. However, nanomicelles should also disassemble near to tumor-tissue of after entering into the cells. Therefore, to check the pH-sensitive disassembly of the prepared CTX conjugated nanomicelles, particle size and PDI were measured in water (pH 7.0), PBS (7.4) and SAB (pH 5.0). As shown in Figure S4, after 12 h incubation in water, the particle size and PDI were changed from 95 to 104 nm and 0.322 to 0.328 for CNM-1; and 94 to 104 nm and 0.315 to 0.338 for CNM-2, respectively. In PBS, the particle size and PDI were changed from 96 to 112 nm and 0.307 to 0.417 for CNM-1; and 95 to 106 nm and 0.315 to 0.337 for CNM-2, respectively.

In case of SAB, the particle size and PDI of both the nanomicelle systems were increased significantly after incubation for 12 h. The size of CNM-1 was increased from 107 to 166 nm and PDI was increased from 0.291 to 0.539. Similarly, and the size of CNM-2 was increased from 103 to 350 nm while PDI changed from 0.318 to 0.927 (Figure S4). These observed particle size and PDI results suggested the prepared nanomicelles were stable in water and PBS but highly unstable in SAB which could be due to the rapid hydrolysis of pH-sensitive linkers at acidic pH.42 The hydrolysis of pH-sensitive linkages at acidic pH led breaking of the bonding between F68 and CTX and dissembling of the micelles which resulted in increased size and polydispersity. While comparing two nanomicelles systems, the higher particle size and PDI were observed within 6 h with CNM-2, indicating that cis-aconityl linkage is rapidly hydrolysed than succinyl linkage. To further confirm the hydrolysis of linkage and dissembling of nanomicelles, TEM analysis was performed. The results of change in the morphology of the CNM-1 and CNM-2 after incubation for 12 h in water, PBS and SAB are shown in Figure 3 which provided more insight into the effect of media or pH-stimulation on the CTX nanomicelles. The images indicate distinct morphology changes with disintegration of the nanomicelles. In water and PBS, the nanomicelles were well intact after the 12 h of incubation. However, after incubation in SAB, nanomicelles were distorted due to the rapid hydrolysis of pH sensitive linkers in CNM-1 and CNM-2. These results clearly indicated that the cis-aconityl-linked CNM-2 more likely cleavable at endosomal pH environment while stable in normal physiological environment.
Figure 3. Transmission electron microscopic images of nanomicelles after incubation for 12 h in water; (a) CNM-1 in water, (b) CNM-2 in water, (c) CNM-1 in PBS, (d) CNM-2 in PBS, (e) CNM-1 in SAB and (f) CNM-2 in SAB.

3.5 In-vitro hemolytic toxicity

In order to verify the biocompatibility of the synthesized nanomicelles, hemolytic toxicity assay was performed. As shown in Figure S5, the nanomicelles prepared with synthesized conjugates did not show any sign of disruption of RBCs membrane and release of haemoglobin. Quantitatively, the observed hemolysis percentage was less than 1% at the tested concentrations and confirmed that the prepared both nanomicelles i.e. CNM-1 and CNM-2 were biocompatible and could be used as a safe carrier for CTX.

3.6 In-vitro CTX release study

The CTX release behaviour of CNM-1 and CNM-2 was assessed by dialysing the formulations at 37 °C in sodium acetate buffer (SAB, pH 5.0) and phosphate buffered saline (PBS, pH 7.4). As shown in Figure 4, the release of CTX from CNM-1 and CNM-2 was greatly affected by the environmental pH 5.0. Approximately 46% and 33% of CTX was released from CNM-1 within 24 h in SAB pH 5.0 and PBS pH 7.4, respectively. Similarly, about 75% and 37%, of CTX was released from CNM-1 within 24 h in SAB pH 5.0 and PBS pH 7.4, respectively. Similarly, about 75% and 37%, of CTX was released from CNM-2 within 24 h in SAB pH 5.0 and PBS pH 7.4, respectively.

Figure 4. Cumulative release of CTX from F68-SA-CTX conjugate based nanomicelles (CNM-1) and F68-CAA-CTX conjugate-based nanomicelles (CNM-2) in sodium acetate buffer (pH 5.0) and phosphate buffer saline (pH 7.4) at 37 °C.

Thus, CTX was released faster from the nanomicelles acidic buffer as compared to physiological buffer PBS. These results clearly indicated that synthesized CTX conjugates are acid-sensitive and more likely cleavable at the acidic endosomal compartments or tumor while remaining stable in a physiological environment at pH 7.4. Further, comparing the release of CTX from nanomicelles at acidic pH, it was found that CTX was released more from CNM-2 (75%) than from CNM-1 (46%). It suggested that the cis-aconityl linkage is more acid sensitive than succinoyl linkage. Also, these results were in agreement with those observed in stability studies.

3.7 In vitro cytotoxicity

The cytotoxic concentrations of pure CTX, CNM-1 and CNM-2 were determined against PC3 human prostate cancer cells lines by MTT assay at varying concentrations (10,000-0.1 nM) after 72 h exposure. The concentration of the compounds causing 50% inhibition of the cell growth (IC50) was determined (Figure 5a). It was observed that the IC50 concentration for all the pure CTX, CNM-1 and CNM-2 was found to be 0.75 nM, 2.17 and 1.97 nM, respectively (Table 1). These results proved the CTX nanomicelles formulations significantly control the growth of human prostate cancer cells and bioconjugation of CTX to F68 does not considerably affect the biological activity of CTX.

Table 1. IC50 (nM) concentrations of pure cabazitaxel (CTX), F68-SA-CTX conjugate nanomicelles (CNM-1) and F68-CAA-CTX conjugate nanomicelles (CNM-2) against 2D human prostate cancer (PC3) cell line.

| Sample     | IC50(nM) |
|------------|----------|
| Pure CTX   | 0.75 ± 0.09 |
| CNM-1      | 2.17 ± 0.30 |
| CNM-2      | 1.97 ± 0.42 |

Figure 5. Cytotoxicity studies on 2D and 3D cells (a) The effect of concentration of pure CTX, CNM-1 and CNM-2 on PC3 cells after 72 hours of exposure. (b) Effect of complexes on PC3 spheroid formation. PC3 cells were grown in ULA cell plates and was treated with IC50 concentration of the pure CTX, CNM-1 and CNM-2. (d) Calcein AM and propidium iodide was added to cells after 72 h and was captured to observe the number of live and dead cells. Quantitative analysis of the spheroids formed were carried out by the addition of ell titer glow reagent to the cells to observe the corresponding luminescence. Scale bars are 100 μm.
3.8 Effect on 3D Multi cellular Spheroids

The 2D MTT assay results was further extended to the assessment of cytotoxicity of CTX nanomicelles in a 3D spheroid system. Due to the close resemblance of the 3D spheroid system with the tumour microenvironment, the viability assays was carried out in 3D multicellular spheroids to assess the IC50 values of the pure CTX, CNM-1 and CNM-2.44 Live dead staining was carried out on spheroids after the treatment with CTX, CNM-1 and CNM-2. The CNM-1 and CNM-2 showed comparable amount of cell death while CTX displayed the lowest number of cell death. Quantitative measurements of cell death in spheroids were also analysed with the help of cell titer glow assay as shown in Figure 5b. CNM-2 has significantly higher cell death percentage (64.54%) at 100 nM as compared to CTX (41.6%) and CNM-1 (46.6%) (Figure 5c). The CNM-2 demonstrated higher cell death rates as compared to other CTX and CNM-1 consistently for all concentrations thus resisting the higher efficacy of the CNM-2 in inhibiting cancer cell growth and proliferation.

Table 2. IC50 (nM) concentrations of pure cabazitaxel (CTX), F68-SA-CTX conjugate nanomicelles (CNM-1) and F68-CAA-CTX conjugate nanomicelles (CNM-2) against 3D spheroids human prostate cancer (PC3) cell line.

| Sample     | IC50 (nM) |
|------------|-----------|
| Pure CTX   | 181.43 ± 11.2 |
| CNM-1      | 177.05 ± 10.5 |
| CNM-2      | 52.55 ± 3.7  |

3.9 Cell cycle analysis

The nanomicelles were investigated in terms of their influence on the cell cycle progression for a better understanding of the mode of action elicited by the CTX, CNM-1 and CNM-2 on cancer cells.45,46 The distribution of treated and untreated (control) cells in different phases of cell cycle is shown in Figure 6. The findings clearly showed the evidence of G2/M cycle arrest with the percentages increasing from 30.9% for untreated cells to 41.3% for CNM-1 and 40.6% for CNM-2. These results were well correlated with the previously published reports.46 It is also noteworthy to observe the simultaneous reduction in the G0/G1 phase values from 57.43% for untreated cells to 37.9% for CNM-1 treated cells and to 38.44% for CNM-2 treated cells. Thus, the results suggested that the cell cycle arrest mechanism of CTX was not altered in nanomicelles formulations but the activity was increased.

3.10 Nuclear morphological changes

To investigate the effect of the compounds on the nuclear morphology, Hoescht 33342 staining was performed and observed the considerable morphological changes in the nuclear chromatin and DNA condensation.47 The cells were treated with the CTX, CNM-1 and CNM-2 at IC50 concentration for 48 h before the addition of the nucleus staining dye. The changes were observed under the microscope revealing the distinct apoptotic characteristics such as cell shrinkage, bright condensed nuclear changes with CNM-1 and CNM-2 while the nucleus of control cells did not show stronger blue fluorescence indicating the non-apoptotic cells (Figure 7).

![Figure 7. Pure cabazitaxel (CTX), F68-SA-CTX conjugate nanomicelles (CNM-1) and F68-CAA-CTX conjugate nanomicelles (CNM-2) induces apoptosis in PC3 cells as depicted by Hoescht 33342 shows the red arrows pointing to the condensed DNA strands in the nucleus indicating apoptosis. Control cells did not show any apparent changes in the nuclear condensation leading to a uniform intensity image while the free CTX, CNM-1 and CNM-2 treated cells showed the presence of intense blue spots indicating the onset of apoptosis. Scale bar: 50 μm.](image)

3.11 Annexin V-PI staining

In addition to the identification of apoptosis through nuclear staining, Annexin V-FITC/PI staining was performed to quantify and to differentiate between the early apoptotic, late apoptotic and necrotic cells.48 The cell positively stained only for Annexin V-FITC were majorly early apoptotic cells while the cells stained for both PI and Annexin V-FITC were considered as late apoptotic cells. The fourth quadrant consisting solely of PI staining comprises of necrotic or dead cells. It is evident from the flow cytometry results as seen in Figure 8 that CNM-2 has the highest percentage of total apoptotic cells (39.2%). CTX and CNM-1 showed almost similar effect with 26.1% and 28.9% of total apoptotic cells, respectively.
3.12 ROS induction analysis

Generation of reactive species (ROS) is one of the paramount phenomena which has been linked to various anti-proliferative processes. Increase of the ROS levels in the mitochondria eventually results in elevated oxidative stress which, in turn, damages the mitochondrial membrane leading to apoptosis.\textsuperscript{69-71} The ROS generation is examined with the help of carboxy-2,7-dichlorofluorescein diacetate (Carboxy-DCFDA) dye which upon cleavage by intracellular esterases oxidizes to highly fluorescent carboxy-2,7 dichlorofluorescein (Carboxy-DCF).\textsuperscript{52,53} As observed in Figure 9a, CNM-2 showed a sharp increase in the green fluorescence while CTX remained relatively less intense with CNM-1 showing higher fluorescence than CTX but slightly lower than that of CNM-2. This increase in the green fluorescence in cells treated with the CNM-1 and CNM-2 indicates the increase in ROS in the cells and therefore, more conversion of non-fluorescent DCFDA to fluorescent DCF molecules. The quantitative analysis of the intensity (Figure 9b) revealed a 4-fold difference in ROS generation between the untreated and CNM-2 treated cells while the fold difference varied by a factor of 2.9 for CNM-1.

3.13 Measurement of mitochondrial membrane potential (MMP)

The effect of the pure CTX, CNM-1 and CNM-2 on the mitochondrial membrane damage was investigated by the addition of JC-1 staining after the treatment of cells with the prepared formulations. Polarised mitochondria are prominently marked by an orange-red punctuated, fluorescence staining. On depolarisation, the orange-red punctate staining is replaced by bright green monomer fluorescence indicating depolarisation.\textsuperscript{54} The untreated/control cells and CTX-treated cells showed intense red fluorescence, indication the formation of aggregates and thus suggesting the intact mitochondrial membrane (Figure 10). The CNM-1 treated cells also showed the formation of few aggregates but with less intensity while CNM-2 treated cells did not show the formation of aggregates. This, in turn, suggests the maximum loss of the membrane potential was caused by CNM-2 followed by CNM-1 and finally by the free CTX. The untreated cells did not show much sign of depolarisation and hence would not enter apoptosis induced by mitochondrial membrane potential loss.

Figure 8. Cell apoptosis determined by flow cytometer analysis against PC3 cells after incubation with pure cabazitaxel (CTX), F68-SA-CTX conjugate nanomicelles (CNM-1) and F68-CA-CTX conjugate nanomicelles (CNM-2).

Figure 9. The effects inflicted by the CTX, CNM-1, and CNM-2 on PC3 cells (a) Cells treated with CTX, CNM-1, and CNM-2, with untreated control cells untreated for the establishment of the baseline ROS levels. The cells were observed for green fluorescence intensities with higher levels of ROS displaying more intense green fluorescence. (b) Quantitative estimation of ROS was done using ImageJ. The green channel intensity was analyzed to measure the fold change in intensity as compared to the control untreated cells. Scale bars: 100 \( \mu \text{m} \).

Figure 10. Mitochondrial Membrane Potential (MMP) study using JC-1 dye: The addition of JC-1 dye to cells treated with pure CTX, CNM-1 and CNM-2 shows the presence of either orange/red aggregates or diffused, intense green fluorescence depending on the state of mitochondrial potential. The untreated control cells and cells treated with the free drug did not lose much of the membrane potential thus displaying a bright orange/red aggregates in the merged channel. The cells treated with CNM-2 shows no presence of orange/red aggregates with clearly intense, punctate green fluorescence appearance re-insisting the depolarisation of membrane potential leading to apoptosis. CNM-2 treated cells, on the other hand, shows sparsely distributed presence of orange/red aggregates leading to the induction of apoptosis. Scale bars: 35 \( \mu \text{m} \).
4. Conclusions

In summary, we successfully developed a surfactant conjugated and pH-responsive prodrugs-based nanomicelles with two different pH-sensitive linkers i.e. succinoyl linker and cis-aconityl linker. The prepared nanomicelles showed nanoparticle scale size, biocompatibility, pH-dependent stability, controlled drug release, and effective cytotoxicity to the PC3 human prostate cancer cells. The cis-aconityl linker based nanomicelles (CNM-2) showed considerably faster drug release at acidic pH than succinoyl linker-based nanomicelles (CNM-1). CNM-2 also presented notable generation, decrease in MMP and induction of more apoptosis than pure CTX. Thus, the cis-aconityl linker-based conjugated nanomicelles could be a more efficient delivery system for anticancer drugs.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

Authors thank the Central University of Gujarat, Gandhinagar for providing necessary facilities and support. HK acknowledges the Department of Science and Technology (DST), New Delhi for an INSPIRE Faculty Award. AKJ and PK acknowledge the University Grant Commission for Ph.D. fellowships. Authors gratefully acknowledge UGC-DAE Consortium for Scientific Research, Indore, M.P., India, for XRD analysis.

References

1. A. Sharma, S. V. Madhunapantula and G. P. Robertson, Expert opinion on drug metabolism & toxicology, 2012, 8, 47–69.
2. L. Li, W. W. Yang and D. G. Xu, Journal of Drug Targeting, 2019, 27, 423–433.
3. F. Luo, Z. Fan, W. Yin, L. Yang, T. Li, L. Zhong, Y. Li, S. Wang, J. Yan, Z. Hou and Q. Zhang, Materials Science and Engineering: C, 2019, 105, 110107.
4. S. K. Golombek, J. -N. May, B. Theek, L. Appold, N. Drude, F. Kiessling and T. Refaat, Molecular and clinical oncology, 2014, 2, 904–908.
5. S. Liao, S. Yang, J. J. Huang, J. K. Chen, A. W. Lee, J. Y. Lai, D. J. Lee and C. C. Cheng, Biomacromolecules, 2018, 19, 2772–2781.
6. H. Wang, M. C. Sobral, T. Snyder, Y. Brudno, V. S. Gorantla and D. J. Mooney, Biomaterials Science, 2020, 8, 266–277.
7. K. Chen, S. Liao, G. Hou, H. Cai, Q. Gong, Z. Gu and K. Luo, Science China Materials, 2018, 61, 1462–1474.
8. Z. S. Liao, S. Yang, J. J. Huang, J. K. Chen, A. W. Lee, J. Y. Lai, D. J. Lee and C. C. Cheng, Biomacromolecules, 2018, 19, 2772–2781.
9. H. Wang, M. C. Sobral, T. Snyder, Y. Brudno, V. S. Gorantla and D. J. Mooney, Biomaterials Science, 2020, 8, 266–277.
10. S. Kaur, C. Prasad, B. Balakrishnan and R. Banerjee, Biomaterials Science, 2015, 3, 955–987.
11. G. Kocak, T. Tuncer and V. Büttin, Polymer Chemistry, 2017, 8, 144–176.
12. H. Wang, Q. Huang, H. Chang, J. Xiao and Y. Chen, Biomaterials Science, 2016, 4, 375–390.
13. J. Li, Z.-E. Hu, X.-L. Yang, W.-X. Wu, X. Xing, B. Gu, Y.-H. Liu, N. Wang and X.-Q. Yu, Biomaterials Science, 2019, 7, 3277–3286.
14. Y. Liang, Z. Su, Y. Yao and N. Zhang, Materials, 2015, 8, 379–391.
15. X. Bin Fang, J. M. Zhang, X. Xie, D. Liu, C. W. He, J. B. Wan and M. W. Chen, International Journal of Pharmaceutics, 2016, 502, 28–37.
16. A. K. Jangid, H. Agrawal, N. Gupta, U. C. S. Yadav, R. Sistla, D. Pooja and H. Kulhari, Colloids and Surfaces B: Biointerfaces, 2019, 175, 202–211.
17. M. K. Khoeeinina, M. Esfandaryani-Manesh, H. Behrouz, M. Amini, B. S. Varnamkhasti and F. A. and R. Dinarvand, Macromolecules, 2017, 50, 251–264.
18. Y. Cai, Z. Sun, Y. Yao, F. Xiao, Y. Wang and M. Chen, Drug Delivery, 2016, 23, 2587–2595.
19. Y. Shao, C. Zhang, Y. Yao, Y. Wang, B. Tian, X. Tang and Y. Wang, European Journal of Pharmaceutical Sciences, 2014, 52, 1–11.
20. K. Chen, S. Liao, G. Hou, H. Cai, Q. Gong, Z. Gu and K. Luo, Science China Materials, 2018, 61, 1462–1474.
21. H. Wang, M. C. Sobral, T. Snyder, Y. Brudno, V. S. Gorantla and D. J. Mooney, Biomaterials Science, 2020, 8, 266–277.
22. S. Kaur, C. Prasad, B. Balakrishnan and R. Banerjee, Biomaterials Science, 2015, 3, 955–987.
23. G. Kocak, T. Tuncer and V. Büttin, Polymer Chemistry, 2017, 8, 144–176.
24. H. Wang, Q. Huang, H. Chang, J. Xiao and Y. Chen, Biomaterials Science, 2016, 4, 375–390.
25. J. Li, Z.-E. Hu, X.-L. Yang, W.-X. Wu, X. Xing, B. Gu, Y.-H. Liu, N. Wang and X.-Q. Yu, Biomaterials Science, 2019, 7, 3277–3286.
26. Y. Liang, Z. Su, Y. Yao and N. Zhang, Materials, 2015, 8, 379–391.
27. X. Bin Fang, J. M. Zhang, X. Xie, D. Liu, C. W. He, J. B. Wan and M. W. Chen, International Journal of Pharmaceutics, 2016, 502, 28–37.
28. A. K. Jangid, H. Agrawal, N. Gupta, U. C. S. Yadav, R. Sistla, D. Pooja and H. Kulhari, Colloids and Surfaces B: Biointerfaces, 2019, 175, 202–211.
29. M. K. Khoeeinina, M. Esfandaryani-Manesh, H. Behrouz, M. Amini, B. S. Varnamkhasti and F. A. and R. Dinarvand, Macromolecules, 2017, 50, 251–264.
30. Y. Cai, Z. Sun, Y. Yao, F. Xiao, Y. Wang and M. Chen, Drug Delivery, 2016, 23, 2587–2595.
31. Y. Shao, C. Zhang, Y. Yao, Y. Wang, B. Tian, X. Tang and Y. Wang, European Journal of Pharmaceutical Sciences, 2014, 52, 1–11.
32. K. Chen, S. Liao, G. Hou, H. Cai, Q. Gong, Z. Gu and K. Luo, Science China Materials, 2018, 61, 1462–1474.
33. H. Wang, M. C. Sobral, T. Snyder, Y. Brudno, V. S. Gorantla and D. J. Mooney, Biomaterials Science, 2020, 8, 266–277.
2017, 5, 532–550.

48  H. Kulhari, D. Pooja, S. Shrivastava, M. Kuncha, V. G. M. Naidu, V. Bansal, R. Sistla and D. J. Adams, *Scientific Reports*, 2016, 6, 1–13.

49  H. Kulhari, D. Pooja, R. Kota, T. S. Reddy, R. F. Tabor, R. Shukla, D. J. Adams, R. Sistla and V. Bansal, *Molecular Pharmaceutics*, 2016, 13, 1491–1500.

50  K. A. Szychowski, K. Rybczyńska-Tkaczyk, M. L. Leja, A. K. Wójtowicz and J. Gmiński, *Environmental Science and Pollution Research*, 2016, 23, 12246–12252.

51  N. Kumar, R. Afjei, T. F. Massoud and R. Paulmurugan, *Scientific Reports*, 2018, 8, 16363.

52  F. Q. Cao, M. M. Yan, Y. J. Liu, L. X. Liu, L. Lu, H. Wang, C. Zhang, H. F. Sun, D. L. Kong and G. L. Ma, *Biomaterials Science*, 2018, 6, 473–477.

53  B. Yang, K. Wang, D. Zhang, B. Sun, B. Ji, L. Wei, Z. Li, M. Wang, X. Zhang, H. Zhang, Q. Kan, C. Luo, Y. Wang, Z. He and J. Sun, *Biomaterials Science*, 2018, 6, 2965–2975.

54  S. Ramesan, A. R. Rezk, C. Dekiwadia, C. Cortez-Jugo and L. Y. Yeo, *Nanoscale*, 2018, 10, 13165–13178.
GRAPHICAL ABSTRACT

- pH-sensitive
- Biocompatible
- Sustained drug release
- Higher cytotoxicity in real tumor 3D environment
- Decrease in MMP

Self-assembled in water