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LETTERS

In-vitro cytotoxic studies of 1-hexylcarbamoyl-5-fluorouracil encapsulated nanogels in BMG-1 cells

Sheetal Budhiraja,[1] Anita Yadav,[2] Rakesh Kumar Sharma,[3*] Rakesh Kumar Sharma[2*]

[1]*Ramjas College, University of Delhi, India.* [2]*Nanotechnology and Drug Delivery Lab, Department of Chemistry, University of Delhi, India*
[3]*SGT University, Gurugram, Haryana, India.*

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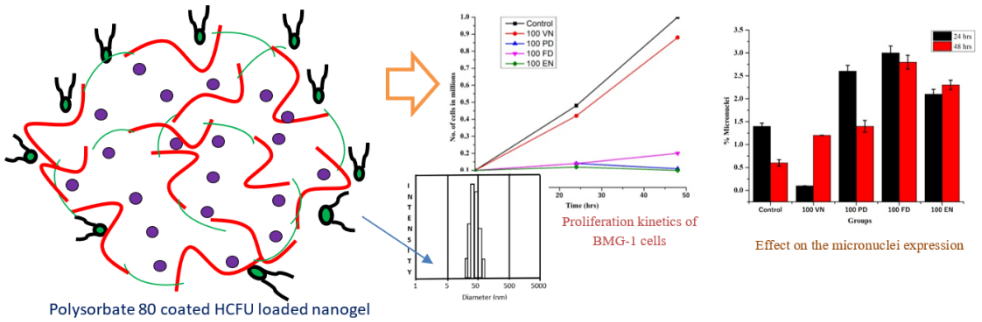
Article

ABSTRACT



5-Fluorouracil (5-FU) has become one of the most widely employed antimetabolite chemotherapeutic agents in the treatment of several cancers including brain, colorectal, breast, head and neck, pancreas and stomach cancers. The variability in 5-FU pharmacokinetics makes oral dosing impractical due to low and unpredictable bioavailability. Carmofur or HCFU (1-hexylcarbamoyl-5-fluorouracil) is a lypophilic-masked analogue of 5-FU. We present studies on encapsulation of HCFU into the nanogels synthesized through copolymerization of N-isopropylacrylamide (NIPAAM) and N-vinylpyrrolidone (VP) having hydrophobic core and hydrophilic shell by cross-linking with N, N'methylenebisacrylamide. This enables easy entrapment and retention of HCFU inside the hydrophobic core of the nanoparticles. A comparison of the therapeutic efficacies and cell sustainability of 5-fluorouracil, HCFU, HCFU loaded nanogels and unencapsulated nanogels as studied in BMG-1 Cells, is reported.

Keywords: Anticancer drug, Prodrug, Nanogel, Cytotoxicity, BMG-1 Cells,

INTRODUCTION

Replacement of the hydrogen atom in position 5 of uracil by a small sized atom of fluorine, by Heidelberger et al.[1], resulted in the synthesis of an important antimetabolite agent named 5-fluorouracil (5-FU). It was designed to occupy the active sites of enzyme targets thereby blocking the metabolism in malignant cells.[2,3] 5-FU has become one of the most widely employed antimetabolite chemotherapeutic agents in recent decades. It has been used as a first line antineoplastic agent in the treatment of several cancers such as brain, colorectal, breast, head and neck, pancreas and stomach cancers.[4–9] The activity of 5-FU is markedly limited by its rapid degradation into 5,6-dihydro-5-fluorouracil (FUH1) via the action of the cytosolic enzyme. It has been demonstrated that this enzyme deactivates more than 80% of the injected dose of 5-FU. The bioavailability of 5-FU is unpredictable. One of the challenges of cancer research is the development of prodrugs of 5-FU that diminish or circumvent some of these disadvantages: reduction in toxicity by avoiding certain routes of degradation (prodrug not being a substrate for the enzymes of degradation) or by targeting the tumor site (prodrug that liberate the active principle selectively in tumor cells). Carmofur or HCFU (1-hexylcarbamoyl-5-fluorouracil), a lypophilic-masked analog of 5-FU has been developed in the National Cancer Centre by the reaction of hexylisocyanate with 5-fluorouracil.[8] HCFU is an inhibitor of acid ceramidase with an IC50 of 79 nM for the rat enzyme. In blood, the lipophilic hexyl group helps it reach tissues or a tumor; and it moderately decomposes into 5-FU or other metabolites in tumor cells where pH is neutral. The hydrophobicity helps a drug molecule to penetrate cell membrane in cases when the molecular target for the drug is located intracellularly.[3] Therapeutic applications of hydrophobic agents are known to be associated with some serious problems because the low water-solubility frequently results in poor absorption and bioavailability. On the other hand, low solubility in water is a characteristic of many drugs, including anticancer agents. Ideal drug carriers for parenteral administration are expected to be biodegradable, have small particle size, possess high loading efficiency, demonstrate prolonged circulation, and, should release the drug at the intended site of action. The development of drug carriers for

*Corresponding Author: Dr. R.K. Sharma (SGT), Prof. R.K. Sharma (DU)
Tel: +919811411632 (RKS-SGT); +919310050453 (RKS-DU)
Email: rksharmadrl@yahoo.com (RKS-SGT), sharmark@chemistry.du.ac.in (RKS-DU)

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HCFU meeting all these requirements still presents a challenging problem.[10] To overcome the poor solubility of HCFU, certain micelle-forming amphiphilic molecules are proposed to be used as drug delivery carrier.[11] In general, micelles are spherical colloidal nanoparticles spontaneously formed by many amphiphilic molecules in aqueous media. Hydrophobic fragments of amphiphilic molecules form the core of a micelle, which can solubilize poorly soluble pharmaceuticals.[12] Hydrophilic parts make the micelle corona. Micelle encapsulation increases the solubility and bioavailability of poorly soluble drugs, increases their stability in biological surroundings, improves their pharmacokinetics and biodistribution, and decreases the toxicity of the drug.[13]. We report studies on encapsulation of HCFU into the nanogels synthesized through copolymerization of N-isopropylacrylamide (NIPAAM) and N-vinylpyrrolidone (VP) having hydrophobic core and hydrophilic shell by cross-linking with N, N'methylenebisacrylamide. This enables easy entrapment and retention of HCFU inside the hydrophobic core of the nanoparticles.[14,15] A comparison of the therapeutic efficacies and cell sustainability of 5-fluorouracil, HCFU, HCFU loaded nanogels and unencapsulated nanogels as studied in BMG-1 Cells, is presented.

METHOD

Synthesis of 1-hexylcarbamoyl-5-fluorouracil

1-hexylcarbamoyl-5-fluorouracil (HCFU) was synthesized using the method already reported.[8] Briefly, hexylisocyanate (19.08g, 0.15 mol) and 5-fluorouracil (13.0g, 0.1mol) were heated on heated mantle in 40ml of pyridine at 90° C for an hour. The reaction mixture was then cooled to room temperature. About 30ml of pyridine were evaporated using rotatory evaporator at 50°C under reduced pressure. To the resulting residue, 50ml of ethanol were added at 55°C. Then the solution was kept at 0-5°C overnight. Crystalline HCFU was thus obtained.

Synthesis of unencapsulated copolymeric nanogels

A random copolymer of NIPAAM and VP was synthesized through free radical polymerization reaction as shown in the flow diagram **(Figure1)**.[15] Water-soluble monomers, NIPAAM and VP, in a molar ratio of 90:10 was dissolved in water. The polymerization was initiated under nitrogen atmosphere using ammonium persulfate (APS) as an initiator. Ferrous ammonium sulfate (FAS) was added to activate the polymerization reaction and also to ensure complete polymerization of the monomers to obtain good yield of the copolymer.[16] Similar polymerization process was used to synthesize the cross-linked polymer chains where N, N'-methylenebisacrylamide (MBA) was used as crosslinking agent. In a typical experimental protocol 90mg NIPAAM, 10µl freshly distilled VP were dissolved in 10ml of water. To crosslink the polymer chain, 30µl of MBA (0.049g/ml) was added in the aqueous solution of monomers. The polymerization was done at 30° C for 24 hours in nitrogen atmosphere. After the polymerization was completed, total aqueous solution of polymer was dialyzed for 4 hours using a spectrapore membrane dialysis bag (12kD cut off) against distilled water. The dialyzed aqueous solution was then frozen in liquid nitrogen and lyophilized immediately to obtain dry powder for further use. Since the copolymer formed was cross-linked using MBA, it no longer remained a micelle and hence named as nanogels.

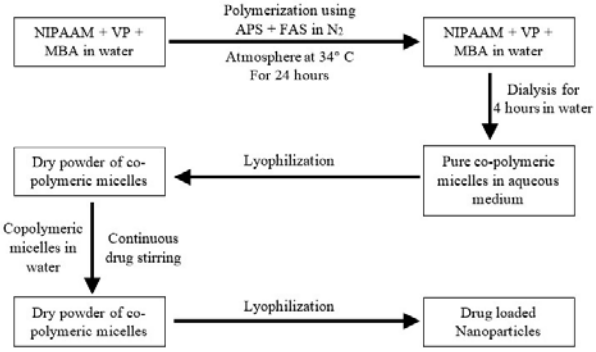

Figure 1. Flow chart for the preparation of copoly (NIPAAM-VP) nanogels where NIPAAM: N-isopropylacrylamide, VP: N-vinylpyrrolidone; MBA: N.N'methylenebisacrylamide, APS: Ammonium persulfate, FAS: Ferrous ammonium persulfate.

Loading of HCFU in the hydrophobic core of nanogels

Nanogels made of cross-linked polymeric micelles may serve as nanoscopic carriers of hydrophobic drugs.[17] In the process of loading, drug was dissolved after the copolymer formation has taken place. Copolymer formed by free radical mechanism has amphiphilic character with a hydrophobic core inside the micelles containing isopropyl groups and hydrophilic outer shell composed of pyrrolidone and hydrated amides in the monomeric units.[18] The copolymer in aqueous solution forms micelles by aggregation of hydrophobic isopropyl groups of NIPAAM moiety present in the polymer. Briefly 100mg of the lyophilized powder of nanogel was dissolved in 10ml of water followed by addition of solution of HCFU in chloroform- methanol mixture (3:1) in the nanogel solution in small amounts and was stirred at room temperature. Up to 1.2% w/w HCFU was encapsulated in the hydrophobic core of the nanoparticles. The drug loaded solution was then lyophilized to give the powder for further use.

Surface coating of nanogels using polysorbate80

Coating with polysorbate80 enhances the uptake of nanoparticles encapsulating various drugs by brain tissue as compared to the control group of uncoated nanoparticles.[19,20] At the moment we speculate that the coating of nanoparticles with polysorbate80 leads to the specific alteration of the surface properties.[16] This new surface then seems to adsorb certain substances from blood that, in turn, induce endocytotic uptake by brain endothelial cells.[21,22] Coating of polysorbate80 on the surface of the nanoparticles was done as follows: 10 mg of lyophilized powder was dispersed in 1 ml distilled water. Aqueous solution of 0.1% w/v polysorbate80 was prepared and 100µl was added to nanoparticulate dispersion for 1%w/w coating respectively.[23–25] Polysorbate80 was added immediately

before the treatment to cells without passing through any lyophilization process.[26]

Characterization of HCFU loaded nanogels

Measurement of size and distribution of nanogels

Average size and size distribution of cross-linked co polymeric micelles was determined by Dynamic Light Scattering (DLS) and morphology of the nanogels was studied using Transmission Electron Microscopy.

Transmission Electron Microscopy (TEM) studies

TEM picture of polysorbate80 coated HCFU loaded nanogels was taken to determine the size and morphology of the nanogels. Samples for the TEM studies were prepared by taking about 10mg of lyophilized powder and dispersing it in 10ml buffer to have a clear solution. The prepared sample solution was put on a formvar-coated grid (1% solution of formvar was prepared in spectroscopic grade chloroform). A clean glass slide was dipped in the formvar solution to make a formvar film on the slide. The glass slides were scratched on the edges and the formvar film was floated on the distilled water. The 2090 mesh copper grids were placed upside down on the floating formvar film. A piece of Whatman filter paper was blotted on the plastic film and lifted out from the distilled water. In this way the plastic-coated grids were prepared. On this grid, a drop of the sample solution was put and allowed to air dry. TEM picture of stained samples of polysorbate 80 coated nanogels loaded with HCFU, (taken in JEOL JEM2000 EX200 model electron microscope) and is shown in **(Figure 2(b)).**

In vitro cytotoxic studies of 5-FU, HCFU, Unencapsulated, Polysorbate80 coated HCFU loaded nanogels in BMG-1 Cells

The cerebral glioma cell line (BMG-1) used to evaluate the cytotoxic effects of 5-FU, HCFU, Unencapsulated, Polysorbate 80 coated nanogels were established in Bangalore, India.

Preparation of cell culture media

Dulbecco's modified Eagle's medium (DMEM) (with L-glutamine and glucose 1g/l, devoid of sodium bicarbonate) supplied as dry powder was dissolved in 900ml Milli-Q water and supplemented with pencillin, streptomycin, nystatin and HEPES N-[2-hydroxyethyl] piperazine-N'[2-ethanesulfonic acid]) at concentration of 50 units/ml, 50 µg/ml, 2 µg/ml and 10mM respectively. After adjusting the pH to 7.4 using 1N NaCl and NaOH to adjust the osmolarity to 310 mOsm, the medium was filter sterilized using 0.22µ filter. The sterility was routinely checked by incubating the medium at 37°C for at least 72 hours. The medium was thereafter stored at 4°C.

Maintenance of cell cultures

Human cerebral glioma cell lines BMG-1 used in the present studies were maintained at 37°C in a humidified CO_2 incubator (5% CO_2, 95% air) in Dulbeco's modified Eagle's medium, DMEM supplemented with 5% fetal calf serum, 50 units/ml pencillin, 50 µg/ml streptomycin sulfate and 2 µg/ml nystatin. Cells were routinely subcultured twice a week using 0.05% trypsin in 0.02% EDTA. The dislodged cells were resuspended in warm complete medium and reseeded.

Treatment Procedure

The groups investigated included 5-fluorouracil, N-hexylcarbamoyl-5-fluorouracil (HCFU), unencapsulated nanoparticles and HCFU loaded nanoparticles at a concentration of 1, 10, 20, 50 and 100µM respectively. Exponentially growing cells were plated in duplicates in 35-mm Petri-dishes at a uniform density of 8000 cells/cm^2, 24 hours before treatment. The formulations were dissolved in Hank's balanced salt solution (HBSS) before filter sterilizing and added to the cells in the required concentration for continuous exposure till 24 and 48 hours. The cells were washed with phosphate buffered saline (PBS-Ca^{2+}, Mg^{2+}free), pH 7.4, and trypsinized at various time intervals for further analysis.

Metabolic Activity

Metabolic activity was estimated using the MTT assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2II-tetrazolium bromide) was dissolved in PBS at a concentration of 5mg/ml and filtered to sterilize and remove small amounts of insoluble MTT. Cells were plated uniformly in 96 well plates (4000-5000 cells/200µl). After a 24-hour preculture period to ensure attachment, medium was removed. Fresh medium was added and supplemented with appropriate volumes of 5-FU, HCFU, unencapsulated nanogels and HCFU loaded nanogels to a final volume of 200µl. The cells were exposed for 24 and 48 hours continuously with the groups to be analysed. Stock MTT solution (20 µl) was added and the cells were incubated at 37°C in the dark during the last two hours of the drug incubation period. The two-hour incubation period had been standardized for the cell lines used for the studies. The medium was removed and any formazan crystals formed by the cells was dissolved using 150µl of DMSO. The absorbance was read at 570 nm using 630 nm as reference wavelength (Mosmann, 1983) on ELISA reader (Biotech Instruments, Vermont). When the absorbance of a solution exceeded 2.0, the solution was diluted; using DMSO and its absorbance was measured.

Cell Proliferation and viability

Cell proliferation and viability studies were carried out by monitoring the growth as function of time in culture. Cell proliferation is related to the growth in number of cells followed by treatment in comparison to the untreated control and cell viability tells us how active the cells are metabolically. Exponentially growing cells (0.08 X 106 / 2ml) were plated in duplicates in 35 mm petridishes. The cells were treated 24 hours post seeding to ensure proper attachment. After incubating with 5-FU, HCFU, unencapsulated nanogels and HCFU loaded nanogels continuously for 24 and 48 hours, cells were washed with PBS and trypsinized. The dislodged cells were resuspended in PBS and quadruplicate haemocytometer counts of duplicate cultures were performed. Cells were further harvested at regular time intervals and enmurated using the haemocytometer.

Cell Cycle Progression

Cell cycle progression was studied by analyzing the cellular DNA content using flow cytometry. The greater the amount of DNA per cell; the higher the fluorescence intensity due to propidium iodide (PI). The amount of DNA per cell varied depending on the phase of the cell cycle it was present in, during the time of fixation. During interphase (Go/G1) mammalian cells contain a diploid (2n) amount of DNA, while just before and during mitosis (G2/M) cells contain twice the amount (4n) of

DNA. During the intermediate S-phase the amount of DNA is between 2n and 4n. Thus, cells in each of the phases of cycle could be obtained by staining with PI and calculating the area under each curve (G0/G1, S and G2/M) of the histogram. Briefly, a quantitative measure of cell cycle distribution was obtained by flowcytometric analysis of DNA histograms. At indicated times cells cultured in the presence or absence of 5-FU, HCFU, unencapsulated nanogels and HCFU loaded nanogels, were detached by trypsinization and fixed in chilled ethanol and stored overnight at 4ºC. Following fixation cells were washed twice with PBS, and incubated with RNase A (200 µg/ml) for 30 minutes at 37ºC and stained using the DNA intercalating fluorochrome, propidium iodode, PI (50 µg/ml) for 30 minutes at 4ºC at a density of 0.5 – 1.0 x 10^6 cells/ml. Samples were stored in the dark at 4ºC until measurements, which was carried out using an argon ion laser (excitation wavelength 488 nm) based flow cytometer (FACS Calibur, Beckton and Dickinson, USA). Red DNA fluorescence due to PI staining was read in the band above 620 nm and the data (forward scatter, side scatter and DNA fluorescence of single cells) was acquired using the Cell Quest software (version 3.0, Beckton and Dickinson, USA). The percentage of cells in different phases of the cell cycle (G0, G1, S) was calculated from the histogram of the DNA fluorescence with the cell cycle analysis software ModFit (version LT 2.0, Verity Software House, USA).

Cytogenetic Damage

Mitotic death (linked to cytogenetic damage) and interphase death (apoptosis) together account for the cytotoxicity of many physicochemical agents including anticancer drugs, although the relative contributions of the two death processes vary among the type of drugs.[27]. Further, mitotic death linked to the cytogenetic damage is also expressed as chromosomal aberrations in the metaphase and manifest in the form of micronuclei formation in the post mitotic daughter cells, which arise from the residual DNA damage, because of the administration of chemotherapeutic agents. Micronuclei expression was studied in the first post treatment mitosis by arresting cells at cytokinesis. At regular intervals cells treated with 5-FU, HCFU, unencapsulated nanogels and HCFU loaded nanogels were washed with PBS and trypsinized. The dislodged cells were re-suspended in a small volume of PBS and fixed in Carnoy's fixative – methanol:acetic acid (3:1) and stored at 4-8ºC for at least one hour. The fixed cells were re-suspended in a small volume of Carnoy's fixative and then gently air dropped on to precleaned, wet and chilled glass slides and left to air-dry overnight. The cells were stained with Hoechst – 33342 at a final concentration of 10 µg/ml in sodium phosphate buffer ($NaH_2PO_4.2H_2O$) (0.5 M): detergent [citric acid (0.1 M) and Tween – 20 (0.5%)] in the ratio 9:1, final pH 7.4. After washing excess stain with distilled water followed by PBS, the slides were mounted using buffered glycerol (PBS: Glycerol – 1:1) and stored at 4ºC. The stained cells were observed under a fluorescence microscope (Olympus BX 60, Japan) using UV excitation filter and fluorescing nuclei were visualized using a blue emission filter. Cells containing micronuclei were counted from >1000 cells by employing the criteria of Countrymen and Heddle.

Apoptosis (Morphological analysis)

Cells that die by programmed cell death commit suicide actively as the result of activation of a dedicated intracellular program. Often, they appear completely healthy prior to committing suicide. For programmed cell death, the most commonly described pathway is apoptosis. Since apoptosis contribute to the antitumoral drugs induced loss of clonogenicity besides mitotic death, we investigated the effects of 5-FU, HCFU and HCFU loaded nanogels induced apoptosis in BMG-1 cells. Some of the morphological changes associated with apoptosis include cell shrinkage, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies (blebbing). A good correlation has been established between these morphological characteristics and DNA ladder, which is one of the hallmarks of cells undergoing apoptosis. Percentage of cells undergoing apoptosis was analyzed by microscopic examination using fluorescence microscope as well as by flow cytometric analysis of DNA content. Acetic acid:methanol fixed cells were stained with the fluorochrome, Hoechst-33342 (10 µg/ml). After washing excess stain with distilled water followed by PBS, the slides were mounted using buffered glycerol (PBS: Glycerol– 1:1) and stored at 4ºC. The stained cells were observed under a fluorescence microscope (Olympus BX 60, Japan) using UV excitation filter and fluorescing nuclei were visualized using a blue emission filter. Cells undergoing apoptosis were counted from > 1000 cells by employing the criteria of Countrymen and Heddle.

RESULTS

The polysorbate80 coated HCFU loaded nanogels were characterised for their size and morphology before and after lyophilisation in order to know if any aggregation occurs. From DLS measurements, the average diameter was found to be around 50nm at 25°C with narrow size distribution and unimodal pattern as shown in **figure 2(a)**. TEM images show spherical morphology of the nanoparticles having a size of around 45 nm. These techniques confirms that there is no aggregation of the nanoparticles even after lyophilisation.

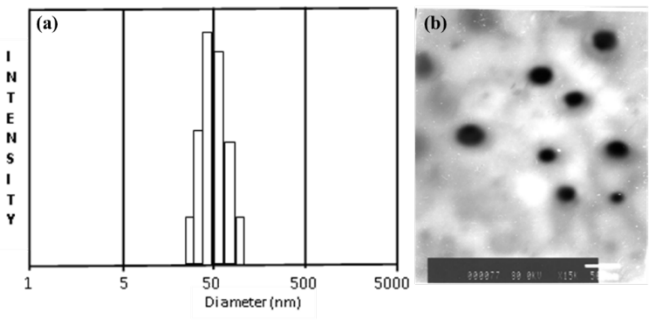

Figure 2. (a) DLS Spectra showing the typical size distribution of the drug-loaded nanogels and **(b)**TEM picture of HCFU loaded nanogels. The picture shows that particles are spherical in shape with an approximate size of around 45nm diameter, which is comparable to the size, obtained from DLS measurements.

A partially hydrophobized polymeric outer shell is capable of inhibiting the intermicellar aggregation of the nanogels during lyophilization and redispersion.[28,29]The colorimetric assay involving the conversion of MTT to formazan, occurs in active mitochondria, and therefore, the reaction takes place only in living cells. The amount of formazan formed reflects the metabolic activity of cells and directly correlates with the number of viable cells. This method has been extensively used for evaluating the effect of drugs on growth and survival of both normal and tumor cells in culture.

BMG-1 Cells treated with the naked drug (5-fluorouracil)[30], prodrug (N-hexylcarbamoyl-5-fluorouacil, HCFU), unencapsulated nanogels and HCFU loaded nanogels. HCFU loaded nanogels showed significant alterations in the metabolic activity of the cells whereas unencapsulated nanogels did not show any alterations in the metabolic activity of the cells which is indicative of the fact that the unencapsulated nanoparticles are not creating any changes in the growth of the cells. The decrease in the metabolic activity when treated with FU, HCFU and HFU loaded nanogels is an indication of either a decrease in the number of cells or the number of mitochondria per cell. The studies were conducted over a wide range of concentrations for FU and HCFU and HCFU loaded nanogels (1-100 µM). The data showed that in case of prodrug and drug there was not much of a difference in the viability at varying concentrations (1, 10, 20, 50, 100 µM) **(Figure 3(a,b))** but in comparison to them drug loaded nanoparticles showed activity at a concentration of 100 µM **(Figure 4(a))**[31,32]. However, compared to the free drug (5-FU) prodrug showed slightly more toxicity towards the cells. Cytotoxicity increases in a dose dependent manner in case of HCFU loaded nanoparticles whereas in case of drug and prodrug no reduction in surviving fraction was observed when the concentration was increased from 1 µM to 100 µM. Thus, cell proliferation, cytogenetic damage and apoptosis for all the four groups were studied at a concentration of 100 µM.

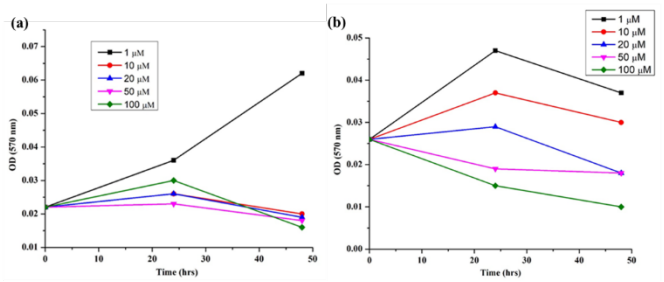

Figure 3. (a) Effect of concentration of HCFU on the metabolic activity of BMG-1 Cells and **(b)** Effect of concentration of 5-fluorouracil on the metabolic activity of BMG-1 Cells.

5-FU, HCFU and HCFU loaded nanoparticles induced both cytotoxic as well as cytostatic effects resulting in a significant loss in the cell counts observed at a continuous exposure time of 24 and 48 hours. The decrease in the number of cells was found to be concentration dependent. At a concentration of 1µM of 5-FU and HCFU there was not a significant difference in the cell number as compared to control and unencapsulated nano-

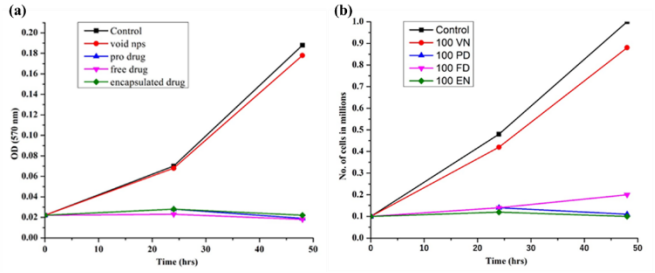

Figure 4. (a) Alterations in the metabolic activity of BMG-1 Cells on continuous exposure for 24 and 48 hours to 5-FU, HCFU, unencapsulated and HCFU loaded nanogels (100µM) monitored by MTT Assay and **(b)**Effect of 5-FU, HCFU, unencapsulated and HCFU loaded nanogels on the proliferation kinetics of BMG-1 Cells followed by treatment (continuous exposure for 24 and 48 hours).

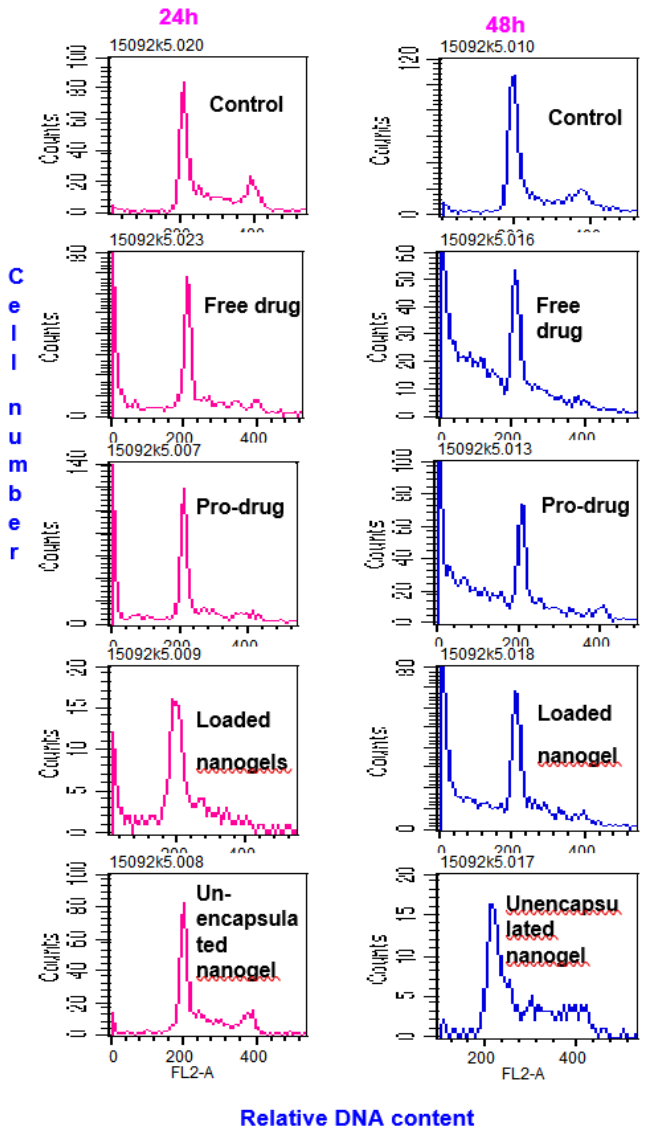

Figure 5. Comparative study of Cell cycle perturbation followed by treatment in exponentially growing BMG-1 cells at different time intervals.

Table 1. Distribution of cells in the cell cycle followed by treatment of BMG-1 cells with 5-FU, HCFU, unencapsulated nanogels and HCFU loaded nanogels

GROUPS	Go/G1%	S %	G2/M %
24 Hours			
Control	47	39	14
5-FU	61	38	1
HCFU	59	39	2
HCFU loaded nanogels	65	35	1
Unencapsulated nanogels	50	39	11
48 Hours			
Control	56	35	9
5-FU	59	40	1
HCFU	55	43	2
HCFU loaded nanogels	57	41	2
Unencapsulated nanogels	58	33	10

-particles but at a concentration of 100 μM **(Figure 4(b))** the cell number decreased to a great extent in case of drug, prodrug and HCFU loaded nanoparticles as compared to untreated controls. However, the unencapsulated nanoparticles had no significant effect on the viability of the cells. This data correlated well with that observed with MTT assay. The re-growth of cells plated following the treatment was also inhibited to some extent in cells treated with drug loaded nanogels. The alterations in the metabolic activity and associated cytotoxicity were observed only when cells were continuously exposed to the groups for 24 and 48 hours. However, the results suggested that the therapeutic effect of the prodrug of 5-fluorouracil is maintained when it is encapsulated inside the hydrophobic core of nanogels.

Since significant reduction in the cell number was observed at a continuous exposure of 24 hours, cell cycle alterations were studied at exposure time of 24 and 48 hours. As seen from **(Figure 5)**, treatment with unencapsulated nanoparticles showed the cell cycle distribution similar to that of control (untreated groups). However, prodrug, drug and prodrug loaded nanoparticles increased apoptosis at 48 hours. **Table 1** suggests that there are no significant changes in different phases of cell cycle but there is an indication of marginal G1/S block with very small increase in cells in G1 phase. No significant changes were observed in S phase of cell cycle. Further a smaller number of G2/M cells was found in cells treated with drug, prodrug and prodrug loaded nanogels. The decrease in number of G2/M cells without any arrest or perturbation in cell cycle with correlated increase in apoptosis implies that G2/M cells or mitotic cells are undergoing cell death following treatment. The data obtained clearly suggests the increased accumulation/availability of 5-fluorouracil released from the core of nanoparticles even at earlier time period of 24 hours of drug exposure to cells. It can thus be concluded that the therapeutic efficacy of the drug is maintained when it is encapsulated in the hydrophobic core of nanoparticles made of copolymers of NIPAAM-VP.

The sensitivity of cells against antitumoral drugs could be determined by the mitotic and interphase (apoptotic) death. The induction of mitotic death was studied by investigating the effect of 5-FU, HCFU and HCFU loaded polymeric nanogels on induction of micronuclei expression in BMG-1cells. Morphological analysis as seen in **(Figure 6)** of cells during follow up showed that a continuous exposure for 24 hours, nanogel formulation including free 5-FU and HCFU have a marginal increase in the number of micronuclei induction than control untreated group and unencapsulated nanogels. However, at 48 hours of continuous exposure not much of difference was observed in the micronucleated expression as compared to 24 hours. These observations indicate that the death of BMG-1 cells caused by free drug, prodrug and prodrug loaded nanoparticles was primarily not mitotic. Since interphase (apoptotic) death also contributes to the cytotoxicity of 5-fluorouracil,[33] we evaluated apoptotic mode of death processes beside mitotic death following various treatments.[34]

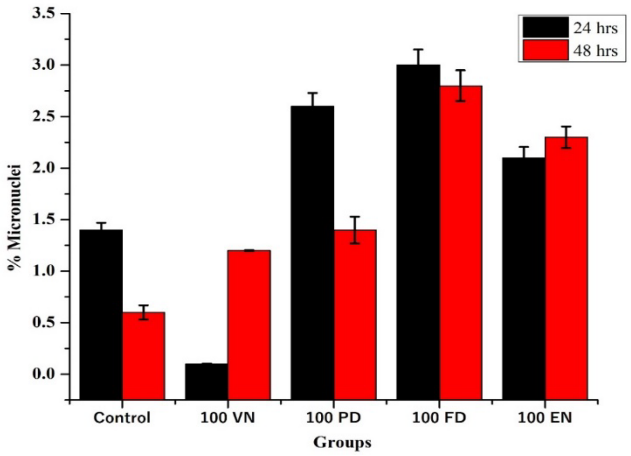

Figure 6. Effect of 5-FU, HCFU, unencapsulated and HCFU loaded nanogels on the micronuclei expression following treatment in exponentially growing BMG-1 Cells.

BMG-1 cells at different post treatment time intervals were processed for both morphological as well as flow cytometric analysis to evaluate the interphase mode of cell death (apoptosis). **Figure 7** showed HCFU loaded nanogels showed higher apoptosis at a continuous exposure time of 24 and 48 hours. On the other hand, control (untreated cells) and unencapsulated nanogels did not exhibit marked apoptosis. The fraction of apoptotic cells in groups composed of 5-FU, HCFU and HCFU loaded nanogels was almost similar at first 24 hours but at a continuous exposure time of 48 hours, an increase in % apoptosis was observed in all the three groups. At a concentration of 100 μM (exposure time 24 hours) around 7.82% cells were undergoing apoptosis when treated with HCFU whereas in case

of free drug and HCFU loaded nanoparticles 6.52 and 6.17% apoptosis was observed respectively. However, when cells were treated for 48 hours continuously % apoptosis in drug, prodrug and prodrug loaded nanogels was found to be 10.9, 11.17 and 9.68% respectively. This may be attributed to their effective cellular penetration and probably sustained release of HCFU from the hydrophobic core of the nanogels. The prodrug is first hydrolyzed to give free drug, which is then slowly released from the nanogels.

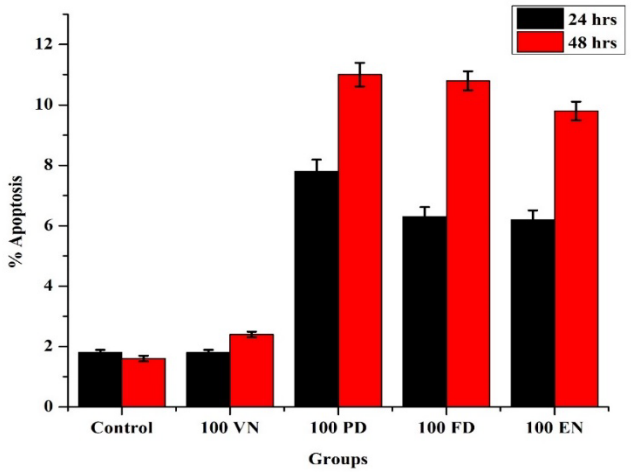

Figure 7. Effect of 5-FU, HCFU, unencapsulated nanogels and HCFU loaded nanogels on the induction of apoptosis studied as a function of post treatment time in BMG-1 Cells.

Discussions

Copolymeric micelles of N-isopropylacrylamide and N-vinylpyrrolidone having hydrophobic core and hydrophilic shell have been synthesized. Nanogels were prepared by cross linking with N, N'methylenebisacrylamide so that HCFU, an amphiphilic molecule, can be easily entrapped and retained inside the hydrophobic core of the nanoparticles. N-isopropylacrylamide was used to form stable micellar aggregates with isopropyl moiety forming the inner hydrophobic core whilst making the copolymer temperature sensitive. N-vinylpyrrolidone renders the hydrogel behaviour to the polymer. To aunencapsulated the dissociation of these polymeric micelles, these are cross-linked in situ to form nanogels. The drug, 5-fluorouracil has been conjugated with hexylisocyanate molecule so that the drug becomes more amphiphilic and can be easily entrapped into the hydrophobic core of nanogel.

Nanogels, prepared by copolymerization of NIPAAM and VP using free radical mechanism, loaded with HCFU, a prodrug of 5-FU was investigated as a potential carrier to sustain the release of anticancer drug in the cells. The cytotoxicity studies were performed in BMG-1 Cells. The nanogels loaded with HCFU were found to be effectively toxic towards the cell lines as compared to the free drug and prodrug. The mode of cell death was not primarily mitotic. This may be because the drug loaded nanogels were able to penetrate intracellularly and the prodrug was slowly being released from the nanogels. The other probability may be that the prodrug was first hydrolyzed to give free drug which is then slowly released from the nanogels. Hence these smart nanogels made of co-polymeric micelles are excellent and well-tolerated drug carriers that could be used for the development of long-acting formulations of anticancer drugs.

Conclusion

Nanogels, prepared by copolymerization of NIPAAM and VP using free radical mechanism, loaded with HCFU, a prodrug of 5-FU was found to be effectively toxic towards the BMG-1cell lines as compared to the 5FU and HCFU. The release of prodrug was slow and consistent from the nanogels. Cytotoxicity increases in a dose dependent manner in case of HCFU loaded nanoparticles where as in case of free drug and prodrug no reduction in surviving fraction was observed. Hence these smart nanogels made of co-polymeric micelles are excellent and well-tolerated drug carriers that could be used for the development of long-acting formulations of anticancer drugs.

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Conflicts of Interest

The authors declare that there is no conflict of interest.

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