RESEARCH ARTICLE

Development of Polymeric Nanopaclitaxel and Comparison with Free Paclitaxel for Effects on Cell Proliferation of MCF-7 and B16F0 Carcinoma Cells

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Abstract

Paclitaxel is hydrophobic in nature and is recognized as a highly toxic anticancer drug, showing adverse effects in normal body sites. In this study, we developed a polymeric nano drug carrier for safe delivery of the paclitaxel to the cancer that releases the drug in a sustained manner and reduces side effects. N-isopropylacrylamide/ vinyl pyrrolidone (NIPAAm/VP) nanoparticles were synthesized by radical polymerization. Physico-chemical characterization of the polymeric nanoparticles was conducted using dynamic light scattering, transmission electron microscopy, scanning electron microscopy and nuclear magnetic resonance, which confirmedpolymerization of formulated nanoparticles. Drug release was assessed using a spectrophotometer and cell viability assays were carried out on the MCF-7 breast cancer and B16F0 skin cancer cell lines. NIPAAm/ VP nanoparticles demonstrated a size distribution in the 65-108 nm range and surface charge measured -15.4 mV. SEM showed the nanoparticles to be spherical in shape with a slow drug release of ~70% in PBS at 38°C over 96 h. Drug loaded nanoparticles were associated with increased viability of MCF-7 and B16F0 cells in comparison to free paclitaxel. Nano loaded paclitaxel shows high therapeutic efficiency by sustained release action for the longer period of time, i increasing its efficacy and biocompatibility for human cancer therapy. Therefore, paclitaxel loaded (NIPAAm/VP) nanoparticles may provide opportunities to expand delivery of the drug for clinical selection.

Keywords: NIPAAm/VP nanoparticles - paclitaxel - sustain drug delivery - cancer cell lines MCF-7 - B16F0

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Introduction

Breast cancer is the second most common leading cause of death in women. Acquired resistance of breast cancer cells is a challenging field in the research. Cancer becomes due to environmental and hereditary factors like chemicals, viruses, or radiation can cause DNA damage that leads to genetic mutations (Tabar and Dean, 2003). From a long time, many strategies have been developed and followed for prevention, diagnosis, and treatment of breast cancer. Mastectomy is a surgical, and preferable option, for treatment that has been replaced by lumpectomy, a radiation therapy (Lerner, 2001). Now, chemotherapy has become the usual treatment for breast cancer patients which helps reduce metastatic tumors (Gurses and Topcul, 2013).

Paclitaxel is chemotherapeutic agent made from Yew trees and mitotic inhibitor that used widely in the treatment of breast cancer. Paclitaxel prevents cancer cells to metastasize by sticking to them while they divide, and stops the division process. It is hydrophobic in nature and given with cremophor EL (polyoxyethylated castor oil) as chemotherapy infusion but the current formulations have severe side effects. Due to its poor solubility (<1 μg/ml) (Goldspiel, 1997), thickness and stickiness, paclitaxel requires a novel drug delivery system.

Recently, thermoresponsive polymeric micelles have attracted extensive research interest as a promising drug carrier for hydrophobic drugs (Kwon and Okano, 1996; Rfsler et al., 2001, Petros and DeSimone, 2010). Novel drug delivery systems for paclitaxel encapsulated nanoparticles have significant proposition in cancer treatment (Wang and Thanou, 2010; Yang et al., 2011; Surapaneniet al., 2012). Poly(N-isopropylacrylamide) (PNIPAAm) with low critical solution temperature (LCST) around 32°C is widely used thermoresponsive polymers with (1995). Polymer micelles are core-shell architecture amphiphilic block copolymers in aqueous

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media. The hydrophobic parts make inner cores and hydrophilic parts make exterior shells. Due to their unique size properties polymeric micelles have high drug loading capacity, and long blood circulation time (Trubetskoy, 1999; Kataoka and Harada, 2001, Verderio et al., 2013). In earlier studies, a number of carriers have been developed such as emulsions (Ludenberg, 1997; Fonseca et al., 2002; Shroff and Kokkoli, 2012), liposomes (Sharma and Straubinger, 1994; Crosasso et al., 2000; Deng et al., 2012), nanoparticles (Bartoli et al., 1990; Chen et al., 2001; Mashinchian et al., 2010; Liechty and Peppas, 2012) and microspheres (Wang et al., 1996; Hamidi et al., 2008) to increase the therapeutic efficacy and better delivery of paclitaxel (Lee et al., 2003).

In this study, we have explored new thermoresponsive polymeric micelles (Ward and Georgiou, 2011; Dhar et al., 2011; Jabbari et al., 2013) PNIPAAm-VP to overcome some of the limitations of currently available polymer micelles. The objectives of this work were to synthesize PNIPAAm-VP nanoparticles and to study the efficacy enhancement on cancer cell lines MCF-7 and B16F0 and size distribution properties of the particles. Nuclear magnetic resonance (NMR), Transmission electron microscope (TEM), Scanning electron microscope (SEM), and DLS techniques were used in the study. The important properties of these particles such as their particle size and stability, encapsulation efficiency, in vitro drug release, and cytotoxicity were also evaluated.

Materials and Methods

Chemicals

Paclitaxel was purchased from Dabur India Ltd., NIPAAm from Across Organics (USA) re-crystallized with N-hexane at 40°C and dried under vacuum, stored at 4°C. VP was purchased from Across Organics (USA) and freshly distilled before use. N-N' methylene bis-acrylamide (MBA) and tetramethyl-ethylenediamine (TEMED) was bought from Sigma (USA) and used directly. Dulbecco's Modified essential medium (DMEM), penicillin, streptomycin, and L-glutamine were purchased from Gibco (Paisley, Scotland, UK). 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), fetal bovine serum, trypsin, 1,10-phenanthroline monohydrate (o-phenanthroline), ethylenediaminetetraacetic acid (EDTA), N,N'-diphenyl-1,4-phenylene-diamine (DPPD), were purchased from Sigma-Aldrich (St. Louis, USA).

Cell culture and treatments

MCF-7 human breast cancer cells and B16F0 Skin Carcinoma cells (National Centre for Cell Science (NCCS) Pune, Maharashtra, India) were routinely grown in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, in a humidified atmosphere of 95% air-5% CO_2 at 37°C. At the treatment stage, the final DMSO concentration was never higher than 0.1%.

Animals

Female Balb/c mice strain, approximately 6-8 weeks old (weights in the range of 30-60 g, were used in this study. The mice were obtained from Central Animal House facility of Hamdard University, New Delhi and were housed in groups of five into well-ventilated room at 22±2°C, under a 12h light/dark cycle. Research on the experimental animals was conducted in Hamdard University by the Indian Ethical Committee (173/CPCSEA). They were acclimatized for one week before the start of the study and were allowed free access to standard laboratory feed (Hindustan Lever Ltd, India) and water ad labium.

Synthesis of NIPAAm/VP nanoparticles

A co-polymeric micelle of NIPAAM-VP was synthesized through free radical polymerization mechanism. NIPAAm and VP monomers are subjected to form random block copolymers with negative zeta potential. In the water, NIPAAm/VP micelles were synthesized having hydrophobic inner core of the isopropyl group of NIPAAm and the hydrophilic shell composed of hydrated amides and pyrrolidone groups. This type of nature of the micelles was used to encapsulate the paclitaxel.

Water soluble monomers, NIPAAm and VP, were used in 80:20 molar ratios and the polymers were cross-linked with MBA. N, N' methylenebis-acrylamidehave two identical unsaturated double bonds and used as an effective cross linking agent in the preparation of NIPAAm/VP micelles during copolymerization. The normal vinyl addition polymerization was done with the monomers. MBA is widely used for the ease of controlling its cross linking properties in the different polymersformulations (Verma et al., 2010).Using a standard experimental protocol, 800 mg NIPAAM, 200 μl VP (also freshly distilled) were mixed in 100 ml of water. To cross-link the polymer chain, 300 μl of MBA (0.049 g/ml) was added in the aqueous solution of monomers. The dissolved oxygen was removed by passing nitrogen gas for 30 min. To initiate the polymerization, TEMED 20 μl (0.5 mol% with respect to the monomers) was added to mixture. The polymerization was fulfilled at 32-35°C for 15h in nitrogen atmosphere. After completing time, the reaction was terminated and solution was dialyzed using cellulose dialyzing membrane (12 kDa cut off). Finally, the polymeric solution is subjected to lyophilization and then a dry powder so obtained. The prepared particles were studied further for characterization.

Characterization of NIPAAm/VP nanoparticles

Dynamic light scattering (DLS): Dynamic light scattering (Photon Correlation Spectroscopy) is a technique used to determine the size distribution profile of small particles in suspension. The technique was performed on "Malvern ZS" instrument at Hamdard University, India. Zeta potential of particles also was determined by same instrument.

Transmission electron microscopy (TEM): The size of the nanoparticles was measured by TEM (Philips Morgagni) at AIIMS, India. Measurements were made using computerized image analyzer and the average size of nanoparticles was noted on a carbon coated grid.

Scanning electron microscopy (SEM): The shape of the

nanoparticles was measured by Hitachi scanning electron microscope (Model No. S-4700) at JNU, India.

1-H Nuclear magnetic resonance (NMR): The NMR spectrum of monomers NIPAAM and VP, as well as void polymeric nanoparticles were taken by dissolving the samples in DMSO as solvent using Bruker 400 MHz spectrometer at Hamdard University, India.

Drug encapsulation and loading efficiency: After the formation of nanoparticles, paclitaxel was loaded in the polymeric micelles through the physical entrapment. 50 mg of the lyophilized powder of NIPAAM/VP nanoparticles was dispersed in 10 ml distilled water. 10mg/ml drug solution of paclitaxel in ethanol was prepared and added to the polymeric solution slowly with vortexing and mild sonication. The drug-loaded nanoparticles are then lyophilized to dry powder for further use.

To calculate the entrapment efficiency (E%) of paclitaxel in NIPAAM/VP nanoparticles, unentrapped free paclitaxel was separated first and then amount of this free drug was calculated using spectrophotometer.

The drug entrapment efficiency was calculated using previously reported equations: ***E%=([Drug]tot-[Drug]free)/[Drug]tot×100***

Drug release: Drug release from the nanoparticles was determined in 1 mL phosphate-buffered saline (50 mM, pH 7.4). As free paclitaxel is insoluble in buffered saline while paclitaxel-NIPAAm/VP were placed in a beaker in horizontal position and kept in incubator shaker for the period of study (38°C, 200rpm). Supernatant (100 mL) was withdrawn from the beaker and replenished with 100 mL fresh buffer at predetermined time intervals. The Paclitaxel concentration in each collected release buffer sample was determined by taking absorbance at 760 nm. Cumulative drug release% was plotted.

Proliferative effect of paclitaxel loaded NIPAAm/VP nanoparticles on MCF-7 and B16F0: The MTT-assay (Mosmann, 1983) was used to evaluate the antiproliferative activities of the paclitaxel loaded nanoparticles. The assay is based on the cleavage of the yellow tetrazolium salt MTT into purple formazan by metabolically active cells, which can be photometrically quantified. For the assays, cells (5×10^3 cells/well in 200 μL of complete DMEM) were placed in each well of a 96 well flat bottom plate. Cells were allowed to adhere for overnight, and then treated with 20 μM of free paclitaxel and paclitaxel loaded NIPAAm/VP nanoparticles for 3, 6 and 12h. After completion of incubation period, 20 μl MTT (5mg/ml) was added to each well for 2h. Following which media was removed and 100 μl of DMSO were added to each well in order to solubilize the formazan. The plate was read using an ELISA reader at a wavelength of 540 nm. The results are expressed as the percentage of viable cells with respect to the control.

Determination of maximum tolerated dose (MTD studies): Maximum tolerated dose is defined as the allowance of a median body weight loss of 15% of the control and causes neither death due toxic effects nor remarkable changes in general signs with in 1 week after administration. MTD for polymeric micelles administered intravenously was investigated in healthy female Balb/c mice. 5 groups were taken for micelles (group 1-5). The doses given were 100mg/kg, 200mg/kg, 400mg/kg, 600 mg/kg and 800 mg/kg were administered to group 1-5respectively. Injection volume was 200 μl when administered through intra venous route for three consecutive days. Mice survival and variation in body weights were observed daily over 30 days in all groups.

Results

Characterization of NIPAAm/VP nanoparticles

Nuclear magnetic resonance (NMR): 1H-NMR data confirmed the synthesis of co-polymeric NIPAAm/VP nanoparticles in Figure 1 showing proton resonance of the vinyl end groups of monomers in the spectrum of the formed co-polymeric micelle. Resonance was observed at the up field region (δ=1.879 ppm). The broad resonance peak at δ=1.011 ppm are from the methyl protons of the isopropyl group and relatively weak signal for proton in methylene group (δ=3.652 ppm). δ=7.269 ppm was observed due to NH proton of N-Isopropylacrylamide group. δ=2.587 ppm peak was recorded for (>CH-) proton next to the carbonyl group. δ=2.483 ppm was observed due to (>CH-) of N-vinyl 2-pyrrolidone group (Table 1).

Dynamic light scattering (DLS): The size distribution of NIPAAm/VP nanoparticles (0.05M) suspended in water was taken by DLS and shown in Figure 2a and 2b. NIPAAm/VP nanoparticles have a negative surface charge (-15.4) in water, thus stabilizing the suspensions via repulsive forces. Zeta potential of the micelles reflects the surface charge. It was used to assess the stability of the nanoparticles. The negative charge on NIPAAm/VP micelles was observed due to bonding of amide group of NIPAAM with water in compression to VP. The figure reveals that the sizes of synthesized NIPAAm/VP nanoparticles are less than 108 nm at 25°C.

Transmission electron microscope (TEM): TEM image of NIPAAm/VP nanoparticles synthesized at 80:20

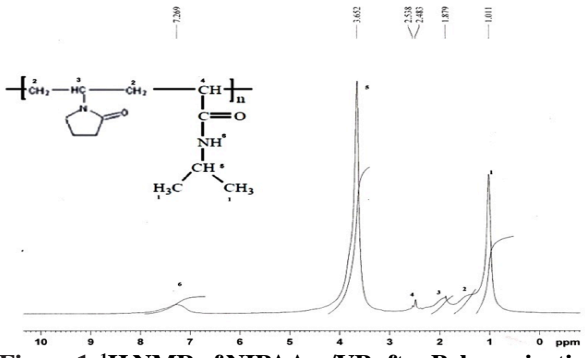

Figure 1. ^{1}H NMR of NIPAAm/VP after Polymerization

Table 1. ^{1}HNMR Peaks Analysis for the Formulation.

S. No.	Peaks (ppm)	Observations
1	1.879	vinyl end groups of monomers
2	1.011	methyl proton of isopropyl group
3	3.652	methylene group
4	7.269	NH proton of N-Isopropylacrylamide
5	2.587	(>CH-) proton next to carbonyl group
6	2.483	(>CH-) of N-vinyl 2-pyrillidone group

molar ratios are shown in Figure 2c. Well-defined 65 nm nanoscale size distribution of highly monodisperse polymeric nanoparticles was obtained.

Scanning electron microscope (SEM): Surface morphology of NIPAAm/VP polymeric nanoparticles is shown in SEM image, revealed the spherical shape and 75 nm size of the formulation in Figure 2d.

Effect of paclitaxel loading on size and zeta potential

The particle size and zeta potential of the blank NIPAAm/VP micelles were measured respectively 108 nm and -15.4 mV by Malvern zeta sizer. When Paclitaxel was loaded their average size was enlarged to 120 nm due to the efficient solubilization of paclitaxel by the micelle core and a slight increase in the zeta-potential of the resulting micelles to -12.9 mV might be explained by the increase in the micelle size because of paclitaxel solubilization in the micelle core and resulting drop in the micelle surface charge density (Figure 3).

Entrapment efficiency and drug release

The highest drug entrapment efficiency of paclitaxel in NIPAAm/VP nanoparticles were achieved 8%.

The in vitro paclitaxel cumulative drug release% from NIPAAm/VP nanoparticles was measured over 96h in Figure 4. An initial release was noted of 9.14% in the first 1 h. In the following 12 hours, cumulative release reached 28.45%, in a sustained manner. Cumulative release reached almost ~70% after 96 hours, showing sustain released ability of the nanoparticles formulation.

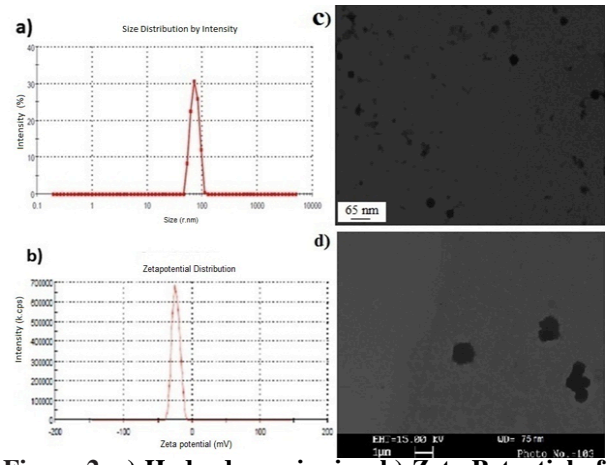

Figure 2. a) Hydrodynamic size, b) Zeta Potential of NIPAAm/VP Nanoparticles. c) TEM Shows Size in nm. d) SEM Showing Shape and Size of NIPAAm/VP Nanoparticles

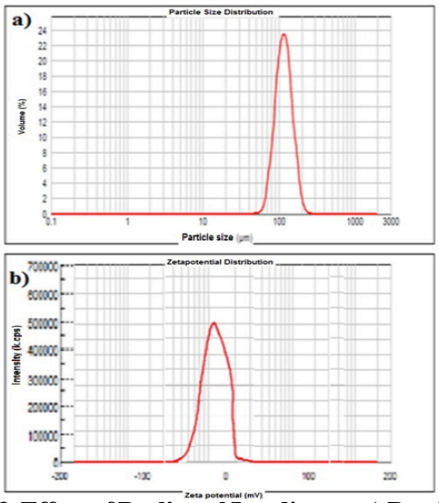

Figure 3. Effect of Paclitaxel Loading on a) Particle size and b) Zeta Potential of NIPAAm/VP Nanoparticles

Proliferative effect of paclitaxel loaded NIPAAm/VP nanoparticles on MCF-7 and B16F0

In the present study we used free paclitaxel and paclitaxel encapsulated nanoparticles for proliferative effect on breast carcinoma (MCF-7) and skin carcinoma (B16F0) cells. As shown in Figure 5a and 5b, treatment of MCF-7 and B16F0 cells with free paclitaxel and paclitaxel loaded nanoparticles resulted in a time dependent inhibition of cell proliferation. As it is evident during the experiment that cell mortality is regularly increasing with paclitaxel at regular interval of 3 and 6h but after attaining a peak value at 12h, it shows constant effect. By repeating experiment with nano loaded drug, it regularly increases the cell mortality at regular interval of 3, 6 and 12h. so it clearly indicates that nano loaded paclitaxel shows high therapeutic efficiency by sustained release action for the

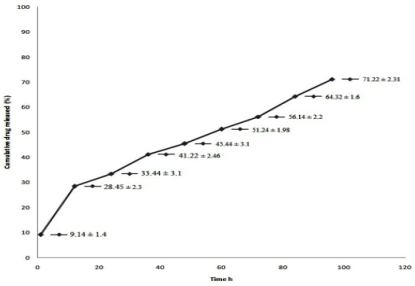

Figure 4. *In vitro* Release of Paclitaxel from NIPAAm/VP Nanoparticles at pH 7.4 and 38°C. Data are Expressed as Mean±SEM

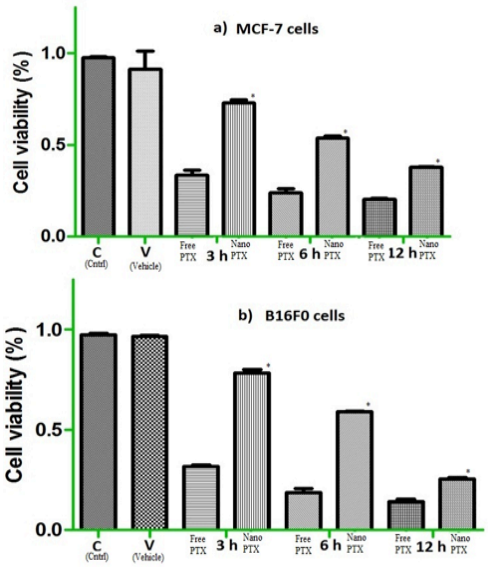

Figure 5.Time Dependent Inhibition of Cell Proliferation by free Paclitaxel, Nanopaclitaxel and Vehicle Alone. Breast Carcinoma Cells (MCF-7) (5a) and Skin Carcinoma Cells (B16F0) (5b). Results are Expressed as the Percentage of Viable Cells with Respect to Control (C). Each Value is the Mean±SEM of The Three Determinations.* Denotes Significant

Table 2. Maximum Tolerated Dose in %

Groups	Weight Loss in %							
	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 15
Group 1 (100 mg/kg)	2.12±1.64	3.33±0.98	2.81±0.9	4.61±1.1	3.44±0.87	4.15±1.4	3.22±0.93	3.86±0.95
Group 2 (200 mg/kg)	6.21±1.8	7.25±1.6	8.01±2.4	8.22±1.46	7.95±1.65	8.65±2.34	8.24±2.87	8.45±1.86
Group 3 (300 mg/kg)	11.23±2.4	12.11±2.1	12.04±1.5	12.25±2.51	11.95±3.1	11.43±1.3	12.62±1.9	13.00±2.82
Group 4 (400 mg/kg)	16.35±1.2	17.35±3.3	19.17±2.08	19.86±1.68	D	0	0	0
Group 5 (500 mg/kg)	19.32±1.1	19.65±2.3	D	0	0	0	0	0

*Group 1-3 not showing lethal toxicity and mortality. Data are expressed as Mean±SEM; Group 4 and 5 showing mortality on the 4th and 8th day.

longer period of time.

Maximum tolerated dose (MTD studies)

Animals showing weight loss exceeding 20% were sacrificed as changes of this magnitude indicate lethal toxicity.The MTD studies for paclitaxel loaded miceller nanoparticles were carried out in non-tumor bearing mice. The MTD for micelles was found to be 600 mg/kg. No apathy was seen in the encapsulated formulations. Mice death was observed on day 8th, group 4, which received the dose of 600 mg/kg of polymeric micelles. Finally group 10 which received the dose of 800 mg/kg showed mortality on day 4th. The data is shown in the Table 2.

Discussion

As defined in the study, a carrier system has been synthesized which enhanced delivery of drug in passively targeting to the tumor site. Scientists have studied various applications of polymeric nanoparticles such as on off drug release regulations, biosensors and intelligent cell culture dishes (Kikuchi and Okano, 1998). In support of polymeric drug delivery systems, many scientists synthesized a new biodegradable tri-block co-polymer of poly (ethylene glycol-b-dl-lactic acid-coglycolic acid)-b-ethylene glycol) (PEG-PLGA-PEG) (Jeong et al., 1999) and poly (L-y-glutamylglutamine)-paclitaxel nanoparticles (Yang et al., 2011). This copolymer showed sol-to gel or gel-to-sol transitions as temperature increases monotonically. Using these materials, they were effectively able to modulate the release kinetics of streptokinase. Thereafter, Poly(N-isopropylacrylamide) (PNIPAAm) has gained importance and has become a well known thermo-sensitive polymer. PNIPAAm polymers are soluble in water at room temperature but being a polymer they have a property that their solubility decreases with increase in the temperature or in other words they undergoes a phase separation at temperatures higher than its lower critical solution temperature (LCST, 32°C). Moreover, NIPAAm being hydrophobic when copolymerized with a hydrophilic monomer results into core shell type nanoparticles (i.e. micelles) (Rfsler et al., 2001). Such self-assembled amphiphilic copolymers have recently been employed to target poorly water-soluble drugs to tumor sites. Small size enables enhanced permeation and retention (EPR) effect due to leaky vasculature and poor lymphatic drainage of the tumors and therefore making passive targeting of the anticancer drugs possible. Active targeting can be achieved by optimizing the fabrication parameters in such a way that LCST is so achieved that the drug is not released in the systemic circulation but inside the tumor.

Chemical shift is defined as the relative difference in resonant frequency compared to a standard signal. Conventionally this is the signal of the compound tetramethylsilane (TMS) for 1H-NMR and a standard fraction of this frequency (Ξ Greek letter Xsi) for all other nuclei.

The size and morphology are the two most important parameters that decide if these particles can be used as carrier for drugs or not. Size below 200 nm makes it ideal for targeting to the tumor as it can undergo passive targeting due to EPR effect. The size and size distribution of the particles formed in deionized water.

The micelles are loaded by dialysis process, which involves solvent exchange mechanism. Both paclitaxel along with the polymer are taken in DMF and these are then poured in a dialysis bag which is finally transferred to water. The molecular weight cut of dialysis membrane was 12 kda and the molecular weight of paclitaxel is 853.

In vitro release of the paclitaxel from nanoparticles was studied under physiological conditions i.e. using PBS at pH 7.4. This initial burst can be attributed to the paclitaxel at the interface of the core and shell as well as for those molecules located at the shell. At temperatures below 37°C, the nanoparticles are well dispersed whereas; at temperatures above it the particles aggregate and settle at the bottom of the dialysis bag. The loss of hydrophilicity/ hydrophobicity, balance of nanoparticles leads to the deformation of the core-shell structure, releasing the encapsulated drug.

In the present study we used free paclitaxel and paclitaxel encapsulated nanoparticles for proliferative effect on breast carcinoma (MCF-7) and skin carcinoma (B16F0) cells. Nano loaded Paclitaxel shows high therapeutic efficiency by sustained release action for the longer period of time. The MTD studies for miceller nanoparticles and paclitaxel (taxol) were carried out in in mice. No apathy was seen in the paclitaxel encapsulated polymeric nanoparticle's formulations.

Hence, hydrophobic paclitaxel-loaded nanoparticles of NIPAAm/VP have been successfully synthesized and characterized by a different technique. The nanoparticles have high stability in the body and release the drug in sustained manner. These types of nanoparticles are highly demanded for their amphiphilic nature, surface properties and encapsulate the hydrophobic paclitaxel which enhance the water solubility of the drug. Drug loaded particles become easily engulfed by the cells and can be targeted specifically to cancer cells. Here, *in vitro* experiment was undertaken, showed significant results which can be

used for further *in vivo* investigation of paclitaxel-loaded NIPAAm/VP nanoparticles.

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