# Chapter 9
# Summary and Conclusion

## 9.1. Summary

In the past few decades, rapid advances in cell and molecular biology have allowed us to develop a better understanding of the pathophysiology of Cancer. Despite tremendous efforts made to conquer cancer in recent decades, it still remains the second leading cause of death worldwide. Breast cancer is difficult to diagnose and treat at an early stage because it grows outward rapidly from deep within the organ and is usually not recognized as a foreign invader by our own body's defense system. Most current anticancer agents do not greatly differentiate between cancerous and normal cells, leading to systemic toxicity and adverse effects. This greatly limits the maximum allowable dose of the drug. In addition, rapid elimination and widespread distribution into targeted organs and tissues requires the administration of a drug in large quantities, which is not economical and often results in undesirable toxicity. Thus, drug targeting has evolved as the most desirable but elusive goal in drug delivery science. It can potentially increase efficacy and reduce toxicity of new and pre-existing drugs by altering their pharmacokinetics and biodistribution and restricting the action of drugs to the treated tissue. Hence, the major challenge is to design drug delivery strategies that deliver the therapeutic agents to the desired intracellular targets based on ability to understand, utilize, modify and exploit membrane trafficking pathways.

Recent advances in nanomaterial technology enable us to control size, shape and surface functionality of nanoconstructs in nanoscale. Because most cancer related biological processes occur at the nano scale, there are many chances to use nanomaterials to diagnose and treat cancers at an early stage. Nanoparticles and liposomes as nanocarriers systems have been used as a physical approach to alter and improve the pharmacokinetic and pharmacodynamic properties of various types of drug molecules. They have been used in vivo to protect the drug entity in the systemic circulation, restrict access of the drug to the chosen sites and to deliver the drug at a controlled and sustained rate to the site of action. In addition, Polymer conjugates, especially those prepared using N- (2-hydroxypropyl) methacrylate (HPMA) have been extensively studied for intracellular and cytoplasmic drug delivery. HPMA conjugated to oligonucleotides via lysosomally degradable spacers were shown to enter the cytoplasm and nucleus of the cells.

Site-specific targeting can be achieved by attaching targeting ligands to surface of nanoconstructs. Various ligands which have been employed include transferrin, folic acid, lectins, RGD petides etc. Many different RGD peptides have been developed. Linear and cyclic RGD peptides, and chemically designed peptidomimetics are currently being tested by researchers. Cyclic RGD peptides are much more potent and more specific than their linear counterparts, and their advantage is their resistance against proteolysis. They have been shown to have selective binding affinity for $\alpha_V\beta_3$ integrin for treatments of human tumor metastasis and tumor-induced angiogenesis. Due to cytoadhesion, cytoinvasion and partial lysosomal accumulation, RGD-mediated drug delivery may provide for improved intracellular availability of conjugated nanocarriers systems.

Docetaxel belonging to the taxane class of anticancer agent is perhaps the most important chemotherapeutic agent against cancer to have emerged over the past several decades. In clinical trials both taxanes are successfully used, mostly against ovarian carcinoma, advanced breast cancer, lung cancer and head/neck cancer. Docetaxel is formulated using polysorbate 80 and ethanol (50:50 v/v). In phase I clinical trials, majority of patients showed acute hypersensitivity reactions with Docetaxel formulated *i.v.* injection. This leads to a need of reformulating Docetaxel with dosage forms to eliminate the toxicity. The objectives of present investigation were development of three types nanoconstructs of docetaxel (PLGA nanoparticles, liposomes and HPMA copolymer drug conjugates), RGD-conjugation onto the nanoconstructs systems, characterization, and evaluation of these nanoconstructs on breast cancer cell lines to understand their role in breast cancer chemotherapy.

The estimation in the nanoparticles, liposomes and *in vitro* release studies for DC was performed by HPLC method. The calibration curve of DC was established in acetonitrile: water gradient system by HPLC at 227nm. The linearity of DC was found to be 0.5-10µg/ml ($R^2$=0.9985). To determine the amount of drug entrapped nanoparticles were dissolved in acetonitrile. The resulting system was centrifuged to remove the precipitated components and the supernant was diluted with the solvent system and subjected to analysis.

The *in vitro* release study was performed using the tube shaking method. At different time intervals, the samples were removed and centrifuged at 25000 rpm and the settled nanoparticles were dissolved in acetonitrile and analyzed for the drug remaining in the nanoparticles as the same method for entrapment efficiency. The drug released was calculated by taking the difference of the drug taken initially and the drug remaining in the nanoparticles.

The estimation of RGD conjugation was carried out using BCA protein estimation. The calibration curve was established at 12.5-1000 µg/ml ($R^2$=0.9935). The amount of PVA associated with nanoparticles was determined by a colorimetric method based on the formation of a colored complex between two adjacent hydroxyl groups of PVA and an iodine molecule. A standard plot for known concentrations of PVA was established at 5-250 µg/ml. ($R^2$=0.9994)

Liposomes were prepared by Supercritical fluid technology using $CO_2$ as an Antisolvent using SPC/drug/HSPC. Process parameters, such as operating temperature, pressure and flow rate of the SCF were found to have significant influence on preparation of liposome (SCF-LP). Hence, process parameters were optimized and kept unaltered in subsequent experiments. In order to optimize process parameters, a Box Behnken Design (BBD) of RSM was used. Fifteen batches of SCF-LP were prepared by SAS process using a 3-factor, 3-level "Box-Behnken" design (BBD) of Response Surface methodology varying three independent variables (Temperature (A), Pressure (B) and $CO_2$ flow rate (C) of the formulation. The Design Expert (Version 7.1, State Ease Inc, USA) program was used for design of experiment and analysis of this second-order model and for drawing of three dimensional response surface and contour plots.

The attempt of liposomes formation was carried out by using mixture of lipids containing both unsaturated and saturated lipid in one liposomal system to improve hydration and encapsulation. The unsaturated and saturated lipids were SPC and HSPC respectively along with cholesterol to design conventional liposomes. The ratio of drug to total lipid was taken as 1:15, 1:20 and 1:25 mole%. The liposomes were characterized for particle size, percent entrapment, vesicle morphology and zeta potential. The optimized process

parameters were kept constant and the formulation parameters were further optimized by the BBD of Response surface methodology.

It has been observed that the increase in CO2 flow rate results in decrease in particle size and increase in the yield of the SC-LP. The possible explanation for this is the high mixing energy and fine atomization of solvent droplets in the stream of SC-CO2 at high flow rate. Generally, a higher mixing energy leads to smaller particles of spherical morphology. Due to the high flow rate, the incoming solvent droplet converts in to fine form and the supercritical fluid removes the organic solvent and leaves behind the small particles in the vessel. As a result of the rapid removal of organic solvent from each droplet, the solute precipitates faster in the high pressure vessel and it is a factor responsible for high yield. It has been found that, as the pressure increases at high temperature the particle size and yield decreases. While at the pressure increases at moderate temperature the particle size decreases but yield increases. The pressure of the SC-$CO_2$ in the high pressure vessels depicts its quantity. This means at high pressure, more amount of $CO_2$ is available in the vessel. The proportion of $CO_2$ directly affects the process of supersaturation for the particle production. The high amount of $CO_2$ hastens the supersaturation process and results in the precipitation of small particles with spherical morphology.

The temperature has significant ($p<0.05$) influence on the yield but non-significant ($p>0.05$) influence on particle size. With increase in temperature along with high pressure, yield decreases might be because of the low $T_g$ (49°C) of the mix lipid system. It is known from the literature that the SC-$Co_2$ lowers the $T_g$ of the polymers. We are anticipating the similar effect responsible for low yield of the final product. The process variables with minimum particle size and maximum yield were considered as optimum and kept constant for further processing.

In our initial trials, we found difficulty in hydration during the preparation of liposomes for docetaxel with saturated lipid (HSPC). Hence, we applied a mix lipid system containing unsaturated lipid (SPC) and saturated lipid with cholesterol. In mix lipid system, the saturated phospholipids and cholesterol provide the rigidity to the membrane and hence improve the stability while unsaturated phospholipid improves the hydration through its polar head groups. The drug entrapment was found to be increase from 72.82±2.18% to 81.4±4.6%

with decrease in drug to total lipid ratio from 1:15 to 1:20 mole % in an optimized batch. However, further decrease in drug to total lipid ratio from 1:20 to 1:25mole% did not have any significant effect on %EE. Hence, 1:20 mole % was considered as an optimum drug to total lipid ratio for maximum drug entrapment.

The Different mol % of DSPE- PEG 2000 was incorporated in the bilayer and effect on percent encapsulation efficiency of DC liposomes was studied. It was found that 6 mol % of DSPE-$PEG_{2000}$-COOH incorporated in liposomes gave optimum entrapment i.e. 79.2±4.4 % among the other molar percentage tried. The percent entrapment was found from 81±4.6 and %, 75.8±2.2%, to their respective molar percent 2 and 8 mole % of DSPE-$PEG_{2000}$-COOH. Entrapment was decreases to 75.8±2.2% when 8 mol % of the polymer was incorporated in the liposome composition. Fall in the percent entrapment was supported by theory that the hydrophobic chain of DSPE-$PEG_{2000}$-COOH would align with the hydrophobic chain of lipids in bilayer and therefore competes with the DC to accommodate in bilayer. There was no significant effect on the particle size observed when the ratio of polymer increased up to 8 mol%. The particle size was found in the range of 260 to 274 nm.

Drug loaded PLGA nanoparticles were prepared by emulsion solvent evaporation method. The process parameters *viz.* homogenization pressure and no. of cycles were optimized for maximum %EE and minimum particle size <250nm. To optimize the process parameters for lowest particle size and highest % entrapment efficiency, the primary emulsion was subjected to different number of homogenization cycles and pressures. The results show that when the pressure was kept constant, the particle size and the % entrapment efficiency decreased with the increase in number of homogenization cycles from one to three. Increase in the pressure from 45 to 125MPa also decreased the particle size as well as the entrapment efficiency. With the decrease in particle size, the total surface area of the particles increases resulting in diffusion of drug from the particles. This may be the reason for decreased entrapment with decrease in particle size. Two cycles at 125MPa was found to be the optimized process parameter. Further increase in the number of cycles resulted in lower particle size which was also accompanied by decreased entrapment efficiency.

Based on the results obtained in preliminary experiments, drug concentration, polymer concentration and PVA concentration were found to be the major variables in determining the entrapment efficiency. The optimized process parameters were kept constant and the formulation parameters were further optimized by the BBD of Response surface methodology. The highest %EE achieved was 72.4 ± 4.6 % with particle size of 240 ± 3 nm for DC-NPs. This was at low level of drug (1%), high level of polymer (10%) and at medium level of PVA (1.5%). The drop in the particle size and higher % EE with the increase in PVA concentration is probably due to the differences in the stability of the emulsions formulated with different concentrations of PVA. Further, the viscosity of PVA solution increases with increasing PVA concentrations. This could result in the formation of a stable emulsion with smaller and uniform droplet size, leading to the formation of smaller sized nanoparticles. There was not much reduction in particle size or increase in %EE when the PVA was increased from 1.5 to 2%. Hence, drug: polymer ratio 1: 10 and 1.5% w/v PVA as an emulsifier were taken as optimized parameters for the preparation of NPs for further studies.
The drug loaded nanoparticles were also prepared by nanoprecipitation technique. The major process parameter effecting the formation of nanoparticles was the speed of the stirring. Evaluation of the variation of the stirring speed was carried out at slow, moderate and high speed. Moderate speed was optimized as the best suitable for the preparation of uniform nanoparticles dispersion. The rate of addition of organic phase to the aqueous was kept constant at 0.5ml/min throughout the entire experimentation. Based on preliminary investigations drug: polymer ratio, PVA concentration in aqueous phase (%w/v) and the ratio of the organic: aqueous phase were found to influence the major variables of particle size and entrapment efficiency. Hence, drug: polymer ratio (represented as polymer concentration, as the amount of the drug was kept constant), PVA concentration in aqueous phase (%w/v) and the ratio of the organic: aqueous phase (represented in decimal form) were kept as independent variables to find optimized condition to obtain optimum particle size (<250nm) with highest entrapment efficiency (dependent variables).

The particle size and entrapment were strongly influenced by the independent variables. The increase in the amount of PLGA (keeping the drug amount constant) resulted in the increase in the particle size of the nanoparticles. The viscosity of the organic phase in

which the PLGA is dissolved appears to affecting the nanoparticles size due to hindrance in rapid dispersion of PLGA solution into the aqueous phase and resulted increase in the droplet and nanoparticles size. Increasing the polymer amount also increased the entrapment efficiency may be due to increase in drug entrapping polymer and due to the decrease in the diffusion of the drug towards the aqueous phase.

Increase in the PVA concentration led to increase in the particle size of the nanoparticles. This increase in the nanoparticles size may be due to increase in the viscosity of the aqueous phase thereby increasing the resistance to the diffusion rate of the organic phase. The miscibility of organic phase (acetone) with aqueous phase results in orientation of PVA at the interface of PLGA solution in acetone present as droplets in the system. Increase in PVA concentration also leads to increase in entrapment efficiency, probably due to reduction in diffusion rate of the organic phase in the aqueous phase.

Increase in the ratio of organic phase: aqueous phase leads to decrease in the particle size and entrapment efficiency. The increase in the organic phase ratio leads increased evaporation time causing slower polymer precipitation, due to the increased microenvironment provided by organic phase after dispersing in the aqueous phase, and thereby formation of small particles. Due to the increased evaporation time and slower polymer precipitation, the tendency of the drug to escape in the aqueous phase before polymer precipitation increases leading to lower drug entrapment efficiency.

The optimized batch was subjected to lyophilization using sucrose, mannitol and trehalose as cryoprotectant at 1:1, 1:2 and 1:3 (nanoconstructs: cryoprotectants). The redispersibility of the lyophilized product and particle size of the nanoconstructs was measured after lyophilization. The redispersibility of nanoparticles with sucrose was poor and was only possible after sonication and show significant increase in particle size. The increase in the particle size could have been due to the cohesive nature of the sucrose. With mannitol, the redispersion was possible only after vigorous shaking and the particle size of the NPs increased on lyophilization. This effect may be due to the low solubility of mannitol in water i.e. 0.18 part of mannitol soluble in 1 parts of water. With trehalose as cryoprotectant, the lyophilized nanoparticles were redispersed easily and the increase in particle size was not significant as indicated by Sf/Si of 1.03 and 1.04 for 1:3 of

nanoparticles: trehalose and liposomes: trehalose, respectively. Hence, trehalose at a ratio of 1: 3 (nanoconstructs: trehalose) was used as cryoprotectant for lyophilization of optimized batch of nanoconstructs for further studies.

The Cyclic RGD peptide was conjugated to the surface of the PLGA nanoparticles by using a two step process. In the first step, the nanoparticles were activated using a sulfoNHS/EDC and in the second step the $NH_2$-PEG-COOH was attached to the activated nanoparticles. The RGD was attached to pegylated nanoparticles by the same process with repetition of conjugation steps.

The surface modification of PLGA-NPs with RGD was achieved in two steps using carbodiimide coupling method. This active ester method yields stable amide bonds. As a prerequisite, the polymer has to contain free carboxyl groups at the surface as represented by the H-type of PLGA which are activated by carbodiimide/N-hydroxysuccinimide. In contrast to the activation of carboxyls with only carbodiimide, the presence of N-hydroxysuccinimide yields N-hydroxysuccinimide esters as stable intermediates which rather acylate amino groups of proteins than to be subject of hydrolysis in aqueous medium. The amounts of activating agents (EDAC/NHS) and RGD were optimized to achieve minimum particle size and maximum RGD density on the surface of NPs.

To check the influence of concentration of activating agents on density of surface RGD, 5 mg of NPs were activated with different concentrations of EDAC/NHS, and the density of surface RGD and particle size of conjugated NPs were measured. It was found that at 0.5/0.3 mmol EDAC/NHS, the surface bound RGD was detectable for conjugated NPs of DC. Increase of EDAC/NHS concentration from 1/0.7 mmole to 2/1.5 mmol increased the RGD density of PLGA-DC-NPs from 14.2 ± 3.7 to 26.4 ± 3.5 μg/mg of nanoparticle surface and further increase in the concentration of EDAC/NHS did not increase the RGD density significantly. The presence of the peptide on the surface, as well as the loss of the fine particles during processing caused the NPs to increase in mean particle size after surface modification. Therefore 2/1.5 mmole EDAC/NHS was taken as the optimized concentration for the activation of PLGA-DC-NPs for the conjugation process.

To maximize conjugation efficiency, different amounts of RGD solution were added to 5 mg of activated PLGA-DC-NPs and the density of conjugated RGD was measured. For

PLGA-DC-NPs as the amount of RGD was increased from 0.5 to 4 mg, the density of conjugated RGD /NPs increased from 6.2 ± 1.7 µg/mg to 30.2 ± 4.5 µg/mg. However, the % conjugation efficiency was found to be insignificant with increased amounts (4mg) of RGD. Therefore, 2mg was taken as the optimized amount of RGD to be used for conjugation of 5 mg of PLGA-DC-NPs.

Mean particle size and zeta potential of PLGA-DC-NP and PLGA-DC-RGD-NP were found to be 210.3 ± 2.7nm and -38.4 ± 2.6mV and 230.7 ± 2.3nm and -10.2 ± 2.8mV respectively. Increase in the particle size after RGD conjugation was due to RGD conjugated. The %EE for PLGA-DC-NP and PLGA-DC-RGD-NP was found to be 71.6 ± 2.4 % and 58.4 ± 1.6 respectively. The reduced drug entrapment efficiency for PLGA-DC-RGD-NP may be due to dissociation of the drug on the surface of PLGA-DC-NP during the RGD conjugation process.

Similarly, the particle size and zeta potential for LP-DC, LP-DC-PEG and LP-DC-RGD were analyzed. Mean particle size and zeta potential of LP-DC & LP-DC-PEG were found to be 260.4 ± 4.6nm& 269.2 ± 2.8nm and -28.7 ± 1.3 & -27.2±1.8mV respectively. While the LP-DC-PEG-RGD demonstrated 278.6±3.4nm particle size and -11.6±0.4mV zeta potential. The %EE for LP-DC, LP-DC-PEG and LP-DC-PEG-RGD was determined to be 72.8 ± 2.2 %, 67.9 ± 3.1% and 64.5±1.5%, respectively.

The release studies of docetaxel from the optimized nanoparticles batch were conducted in phosphate buffer pH 7.4 + 0.1%w/v polysorbate-80. For PLGA-DC-NP there was an initial burst release of about 19% in 12hrs and then there was a lag phase and about 80% release resulted in 21 days. The unconjugated nanoparticles showed a high initial burst which can be attributed to the immediate dissociation and dissolution of drug adhered on the surface and located near the surface of the NPs. The burst effect was absent in PLGA-DC-RGD-NP and the release in 21 days was found to be about 60%.

For docetaxel encapsulated liposomes, it was found that both formulations produced an initial burst release in which docetaxel release was more than 10 % and 8 % for pegylated and non-pegylated liposomes, respectively, within the initial sampling time (30min). The burst release in both forms of liposomes is related to release of surface adsorbed drug. After the initial burst release, a constant drug release was found and maximum of 75% and 60%

drug was released in 48hr of time period with pegylated and non pegylated liposomes respectively. Faster release in pegylated liposomes may be due to the fast hydration process of PEG molecules on the surface of the particles.

Transmission Electron Microscopy and Scanning Electron Microscopy images of the unconjugated and conjugated NPs and liposomes showed spherical configuration with smooth surfaces. DSC thermogram of plain Docetaxel depicts a sharp melting peak from 169-171°C, indicating the crystalline nature of the drug. The drug in the amorphous form is entrapped in the nanoparticles. For PLGA-DC-NP the peak of docetaxel is absent indicating the conversion of DC into amorphous state during the nanoparticles formulation and thereby entrapped in the PLGA polymer. Similarly, for LP-DC, the peak of docetaxel is absent indicating the conversion of DC into amorphous state during the liposome formulation and thereby entrapped in the liposomes. XRD patterns of the PLGA-DC-RGD-NPs and LP-DC-RGD depict that the formulations were in amorphous forms.

The stability studies were carried out in accordance with the ICH guidelines for drug substances intended to be stored in a refrigerator. The stability of the nanoparticles and liposomes were assessed for physical observation, particle size, zeta potential and the drug content (with respect to the initial) at 5°C ± 3°C for 6M and 25°C ± 2°C/60% RH ± 5% RH for 6 months.

It was observed that unconjugated and conjugated nanoparticles of docetaxel there was no significant change ($P>0.05$) observed in particle size, zeta potential and drug content at 5°C ± 3°C for 6M. The storage of the unconjugated and conjugated nanoparticles of docetaxel at 25°C ± 2°C/60% ± 5% RH, led to increase in the particle size. The increase in the particle size was not significant during the first month, however became significant and more prominent after 2, 3 and 6 months. During our analysis of samples, the polydispersity index of the nanoparticles stored at 25°C ± 2°C/60% ± 5% RH was found to increase as compared to the initial. The increase in the particle size may be due to the absorption of the moisture by the nanoparticles resulting in the coalescence of the small nanoparticles forming particles larger in size.

The nanoparticles were also observed for physical appearance. After 3 and 6 months the physical appearance was also changed, with loss of the free flowing property followed by

the difficulty in redispersibility. Also, the RGD conjugated nanoparticles demonstrated difference in the color than the initial powder. At 6 months the color of the powder was light pink. This could be indicative of the degradation of the surface RGD.

At 25°C ± 2°C/60% ± 5% RH, the zeta potential of the nanoparticles shifted towards the zero for both unconjugated and conjugated nanoparticles. This may be due to the acidic conditions produced due to the degradation of PLGA into lactic and glycolic acid. The lowered zeta potential values also might have contributed toward the aggregation of particles. The drug content of the unconjugated and conjugated nanoparticles was not altered up to 6M at 5°C ± 3°C. However, the drug content was reduced after 6M storage at 25°C ± 2°C/60% RH ± 5% RH. This impact could be due to the moisture absorbed by the nanoparticles upon storage at 25°C ± 2°C/60% RH ± 5% RH, possibly resulting in the degradation of the drug. The initial drug entrapment was found to be 72.6±2.4% and 69.6±3.4% and initial particle size 260.6±2.4 nm, 260.6±2.4 nm for Non-PEGylated and PEGylated liposomes respectively. Reduction in the entrapment after 3 months, were observed 69.2±2.8 (2-8°C) and 61.2±3.8 mg (25°C) while particle size was 269.2±4.4 nm (2-8°C) and 288.2±7.6 nm (25°C) for Non-PEGylated. In case of PEGylated liposomes, minor reduction in the drug entrapment was seen (68.2±2.1 and 61.2±3.8) at 2-8°C and 25°C respectively and increase in the particles size was in the range of 8-12 nm from the initial.

From the above study, we can demonstrate that the unconjugated and RGD-conjugated PLGA nanoparticles and liposomes of docetaxel when stored at 25°C ± 2°C/60% RH ± 5% RH for 6M show instability reflected by change in physical appearance, increase in the particle size, zeta potential and reduction in the drug content. Hence, we can conclusively specify that both unconjugated and conjugated nanoparticles and liposomes of docetaxel were stable and can be stored 5°C ± 3°C for 6M retaining its original formulation characteristics.

*In vitro* cell culture studies were performed on BT-20 and MDA-MB-231cells to assess the intracellular nanoparticles and liposomes uptake, to study the antiproliferative action, to assess the DNA content and to evaluate the mode of cell death by nanoparticles and liposomes with compare to the drug solution. BT-20 and MDA-MB-231 cells were cultured and maintained as monolayer, growing as adherent monolayer in DMEM. DMEM was

supplemented with 10% fetal bovine serum at 37°C in the atmosphere of 5% $CO_2$ and 95% relative humidity.

Intracellular uptake of the nanoparticles and liposomes into the cell was studied by incorporating 6-coumarin (fluorescent dye) into the nanoparticles (PLGA-NP) & liposomes (LP) and further conjugated with RGD (RGD-PLGA-NP & RGD-LP). Quantative intracellular uptake of the nanocarriers was performed to assess the influence of time and concentration. The uptake of the nanoparticles was directly proportional to the concentration of the nanoparticles in the medium. It was observed that the cellular uptake of the nanoparticles increased with increase in the concentration. However, the cellular uptake efficiency was found to be highest at the lowest concentration and found to decrease with increase in concentration. The decrease in the uptake efficiency indicates that the cells might have reached the saturating capacity for uptake. RGD-conjugated nanoconstructs at all concentrations demonstrate 1.6-2.1 folds increased uptake over unconjugated nanoconstructs uptake. The superior uptake of the RGD conjugated nanoparticles could be due to specific active endocytosis process mediated through the RGD adhesion to the integrins.

The results of quantitative uptake studies indicate the uptake of the nanoparticles into the cell. The untreated cells did not demonstrate any fluorescence. The nanoconstructs cell uptake efficiency was found to increase with time from 0.25-4hrs for both the unconjugated and RGD conjugated nanoconstructs. At all time points, the uptake efficiency of RGD conjugated systems were approximately 1.6~2.1 folds higher than the unconjugated systems. At 5hrs there was not much increase in the uptake efficiency suggesting the saturation of uptake with time. Hence, the influence of the concentration was performed by keeping the incubation time at 4hrs for all concentrations.

The uptake of the nanocarriers in the cells was qualitatively confirmed by visualizing under fluorescent microscope. The fluorescent microscopic images showed green fluorescence in the cell cytoplasm and around the nucleus which reflects the internalization of the nanocarriers into the cell. Based on this observation, it is reasonable to believe that the nanoparticles may carry the active drug across the cell membrane into the cytoplasm. The RGD conjugated nanocarriers at all the time points demonstrated higher fluorescence intensity than the unconjugated nanocarriers confirming the results of the quantitative uptake

studies. Also the RGD conjugated nanocarriers show higher number of the cells showing fluorescence than the unconjugated nanoparticles.

The cytotoxicity studies were performed using CCK-8 assay. Cytotoxicity studies were performed at different nanomolar concentration range for free docetaxel and docetaxel encapsulated targeted and nontargeted nanoconstructs. The cytotoxicities of unconjugated and RGD conjugated nanoconstructs were significantly ($p<0.05$) higher than drug solution. The cytotoxicity was found to be dose and time dependent.

The cytotoxicity indicated by $IC_{50}$ values suggests that RGD conjugated nanoparticles at 72hrs are 2.1 and 10 time more cytotoxic than unconjugated nanoparticles and drug solution for BT-20 cell lines. Similarly, for MDA-MB-231 cells, the RGD conjugated nanoparticles are 2 and 4 time more cytotoxic than unconjugated nanoparticles and drug solution.

The RGD conjugated docetaxel nanoparticles produced significantly higher toxicities at all time points, i.e 24, 48, 72hrs, than the drug solution and unconjugated nanoparticles. After 24hrs, RGD conjugated nanoparticles at the higher concentrations showed superior toxicity differences compared to drug solution and unconjugated nanoparticles. An important observation was that with increase in incubation time at 72hrs, the lowest concentration of RGD conjugated nanoparticles showed greater toxicity than drug solution and unconjugated nanoparticles. However, the higher concentrations didn't show much toxicity difference than the drug solution and unconjugated nanoparticles.

The cytotoxicity indicated by $IC_{50}$ values suggests that RGD conjugated liposomes at 72hrs are 1.875 and 8.3 time more cytotoxic than unconjugated liposomes and drug solution for BT-20 cell lines. Similarly, for MDA-MB-231 cells, the RGD conjugated liposomes are 1.82 and 4.7 time more cytotoxic than unconjugated liposomes and drug solution.

Cell cycle analysis was done to determine the growth phase in which the cells were accumulating due to inhibition of cell cycle progression. Docetaxel inhibits cell growth and proliferation primarily through its effect on the cell cycle and induction of apoptosis and necrosis.

In case of free drug, the arrest in G2 phase was observed at lower concentration of 1nM and 2 nM but due to the high amount of debris, the exact percentage of each phase was

difficult to calculate by Modfit software. At 24hrs, the cell cycle arrest in G2 phase was increasing significantly ($p<0.05$) as increase in concentration from 10nM to 30nM for free drug. However, the difference was insignificant ($p>0.05$) at 48hrs between intra concentrations of free drug at 48hrs. It was observed at 48hrs, that with 10nM of free drug solution, the percentage G2 phase arrest was increased significantly compared to 24hrs effect. But the difference was insignificant with 30nM and 50nM of free drug. As shown in table 7.11 the RGD conjugated nanoparticles at the drug equivalent concentration of 5nM showed 51% G2 phase arrest as compared to 30% G2 phase arrest with unconjugated nanoparticles at the same concentration at 24hrs. The block in G2 phase was found to be time and concentration dependent. At 48hrs, there is no significant difference between the effects of conjugated and unconjugated nanoparticles at 10nM concentration. Similarly, RGD conjugated drug encapsulated liposomal system was found to have significantly high arrest of cell cycle as compared to unconjugated system at same concentrations. The cell cycle arrest was found to be concentration and time dependent.

Cell cycle analysis demonstrated that all free DC, PLGA-DC and PLGA-DC-RGD arrested BT-20 cells in the G-2 phase of the cell cycle. However, in cells treated with PLGA-DC and PLGA-DC-RGD, the appearance of populations shown to be in G-2 was found to be more by at least 2 fold as compared to those treated with free DC. Further, the accumulation of cells in G-2 was accompanied by a 7-8 fold decrease in the number of cells in S phase compared to control cells, an indication of DNA replication inhibition. Blank PLGA and RGD peptide alone had no effect on cell cycle progression when applied using the same range of concentrations as PLGA-DC and PLGA-DC-RGD.

The apoptosis and necrosis effect was found to be concentration and time dependent for both types of drug delivery systems. In case of nanoparticles, the RGD conjugated nanoparticles have more apoptotic cell fragments than the unconjugated nanoparticles and drug solution at the same drug equivalent concentrations. At 24 hrs, the treatment of 5nM of drug solution with cells resulted in 13.6% & 3.4 % necrotic & apoptotic cell fragments respectively. While at similar drug equivalent concentration, the PLGA-DC-RGD and PLGA-DC nanoparticles showed 6.4% & 28.3% and 5.1% & 25.6% necrotic & apoptotic cell fragments respectively. At 72hrs, the percent apoptotic cell fragments were significantly high

for targeted nanoparticles as compared to untargeted nanoparticles and drug solution at 5nM concentration. While at 72hrs, the 5nM drug solution had significant high necrotic cell fragments as compared to targeted and untargeted Nanoparticulate systems. The free drug has found to have two mode of action at two different concentrations. At 5nM concentration it produces significantly high necrosis and low apoptosis. While at 10nM concentration the observed effect was reversed i.e. high apoptosis and low necrosis. The necrosis effect was significantly low in case of targeted and untargeted nanoparticles at both concentrations. At 10nM drug eq. concentration, the PLGA-DC-RGD nanoparticles have shown to be significantly high percentage of apoptosis with compared to same concentration of drug solution at all time points i.e. 24hrs, 48hrs and 72hrs. However the difference is insignificant with compared to PLGA-DC nanoparticles. Similar kinds of effects were observed with liposomal systems also.

**9.2. Conclusions**

To conclude, docetaxel encapsulated PLGA loaded nanoparticles and liposomes, and docetaxel conjugated HPMA copolymer were successfully prepared using solvent evaporation, Supercritical $CO_2$ as anti-solvent method and free radical polymerization techniques respectively. Cyclic RGD peptide was attached to these nanoconstructs to impart ability to target breast cancer. The characterization of nanoparticles and liposomes demonstrated small particle size (< 300nm) suitable for intravenous administration, optimum drug encapsulation and prolonged drug release. The intracellular uptake of nanoparticles and liposomes loaded with fluorescent dye 6-coumarin showed significantly higher cell uptake after RGD conjugation of nanoparticles due to the receptor mediated uptake through the RGD receptors located on the BT-20 and MDA-MB-231 breast cancer cells. The HPMA-docetaxel copolymer conjugates were found to have high molecular weight (>30KDa), optimum drug content .Hydrolysis of conjugates by lysosomal enzyme to release drug for pharmacological response was found to be above 50% of conjugated drug when assessed *in-vitro* by Cathepsin B enzyme hydrolysis.

The docetaxel encapsulated liposomes were found to be more effective than PLGA nanoparticles, HPMA copolymer conjugates and free drug solutions at equivalent drug concentrations when assessed for cytotoxicity, DNA content and mode of cell death analysis.

The difference may be attributed to the quick drug release inside the cytoplasm from the liposomes due to the rapid hydrolysis of pegylated lipid membranes. Drug release from PLGA nanoparticles was slow due to the more hydrophobic nature of PLGA and slow biodegradability of the surface ester bonds and hence, had moderate chemotherapeutic efficacy for prolonged period. The docetaxel conjugates with HPMA copolymer with tetrapeptidyl linker ( Gly-Phe-Leu-Gly), cleave by endolysosomal proteases after endocytic uptake by the cells and are stable in circulation. The cleavage of the linkage delays the drug release inside the cell and hence, chemotherapeutic efficacy of the conjugates. But, chemotherapeutic response of the conjugates was prolonged to the maximum extent compared to other nanoconstructs prepared in this investigation. Chemotherapeutic efficacy of RGD conjugation to any of nanoconstructs prepared in this investigation was found to be more than those nanoconstructs without RGD conjugation. All nanoconstructs formulations were found to be stable for six months when stored at refrigerated condition (2-8°C). The in vitro serum drug release data suggest that the nanoparticles release least (6.5%) drug in 24hrs followed by liposomes (35%) HPMA copolymer conjugates (above 50%).

The RGD attachment via a Glycyl- glyceine non cleavable linkage makes drug HPMA conjugates fairly stable in blood circulation and surface density of RGD is also maximum compared to drug nanoparticles and liposomes providing most target specific drug delivery. Moreover the drug release been slower, it provides prolonged chemotherapeutic response and also helps in prevention of development of drug resistance. These benefits made RGD attached docetaxel HPMA conjugates most attractive among the nanoconstructs studied in this investigation. The advantages are also in terms of ease of chemical synthesis of HPMA copolymer conjugates in high quantity and least expensive set of equipment requirement favoring scale up of developed technology as compared to liposomes or nanoparticles. However, more extensive animal experimentation in terms of chemotherapeutic efficacy and side effects are necessary to take up findings of this investigation to clinical evaluations.