Research Article

Nanoparticles & Pegylated Nanoparticles: Development, Comparison and Evaluation of Its *In Vitro* Fate

Anand Mahalwar[1*], Amit Sharma[1], Rajesh Sahu[2] and DS. Rathore[1]

[1]Suresh Gyan Vihar University, Rajasthan, India.
[2]Rayal College of Pharmacy Science, Raipur, India.

ABSTRACT
The present study is aimed to develop and characterize drug (cisplatin) loaded, PEGylated nanoparticles (PEGyNPs) and compare its efficiency with PLGA (drug loaded) nanoparticles (PLGANPs). For this study mPEG (methoxy Polyethylene glycol) and PLGA were polymerized to form copolymer and their synthesis was characterized via ^{1}H NMR spectroscopy. Then both polymers (mPEG-PLGA copolymer & PLGA polymer) were used for preparing PLGANPs and PEGyNPs and then process variables were optimized according to their particle size, polydispersity index (PdI) and entrapment efficiency. Various parameters i.e. Trans Emission Microscopy (TEM), Surface emission Microscopy (SEM), zeta potential and zeta sizer, drug entrapment, drug release of both PLGANPs and PEGyNPs were determined and compared. The PEGyNPs conforms increase in drug entrapment, reduction in drug release rate and burst release. The PEGylated nanoparticles were found suitable for sustained and controlled delivery of cisplatin.

Keywords: PLGA poly (lactide-co-glycolide), Nanoparticles, PEGylated nanoparticles, TEM, SEM.

INTRODUCTION

Every biological level of organization consist a unique mechanism (barriers) to prevent the delivery of therapeutic agents. Some of the potent barriers are, non specificity in targeting, enhanced clearance, selectivity and permeability of biological membranes, metabolizing enzymes and endosomal/lysosomal degradation. To achieve better therapeutic concentration in the site, this mentioned barriers should be overcome (Bareford and Swaan, 2007). For the treatment of cancer, chemotherapy has proved his utilization. But major challenges in chemotherapy are its undesired toxicity to the other cells and development of multidrug resistance (MDR) against anticancer drugs, this limits higher concentration of therapeutic agent on the desired site. Ones the resistance is initiated for the cytotoxic agents it also extends the cross-resistance to wide range of drugs having different chemical structures. And if it gets initiated (cross-resistance) chemotherapy either targeted or none targeted becomes ineffective and its further leads to the increase of resistance (Lee et al., 2008; Fojo and Coley, 2007; Oconnor, 2007; Higgins, 2007).

MDR is characterized by the resistance of the tumor cells to the wide range of chemically unrelated anticancer drugs, one potent reason to develop MDR in cells are activation of ATP-driven membrane pumps and thus increase in efflux mechanism. Some of the identified well-known ATP-driven efflux pump families includes, P-glycoprotein (P-gp), multidrug resistance protein (MRP1), canalicular multi-specific organic anion transporter cMOAT (MRP 2), Breast cancer resistance protein (BCRP) etc., out of this all MDR developed by P-gp are well characterized and focused importance in clinical trail (Mohajer et al., 2007; Sehested et al., 1987; Simon and Schindler, 1994; Cataldo et al., 2003; Lazzarino et al., 1998; Schinkel and Jonker, 2003).

Excipients such as PEG stearates, PEG fatty acid esters, polysorbate and Poloxamers (Class III generation, P-gp inhibitors) has been reported to inhibit the P-glycoprotein *in vitro* but little attention was taken for its *in vivo* characterization (Choi and Shin, 2005; Kabanov et al., 2003).

Nanoparticles (NPs) are attracting major attention as a promising colloidal drug carrier in the recent years. Nanoparticles (NPs), are defined as solid colloidal particles consisting of macromolecular compounds in the size typically from 10 to 100 nm in diameter, they are formulated from a biodegradable polymer in which the therapeutic agent is entrapped, adsorbed or chemically coupled to the matrix

of polymer (Labhasetwar, 1997). They were initially devised as carriers for vaccines and anticancer drugs to limit the off-target tissue toxicity present in conventional methods. NPs can be fabricated from a multitude of materials, including synthetic polymers and biopolymers (proteins and polysaccharides). Drug integration of peptide segments, proteins, and/or small molecules with both targeting and therapeutic abilities into delivery systems in the form of nanoparticulate polymer matrices offers many benefits. These benefits include controlled drug release and protection, prolonged blood circulation times, and many other adjustable characteristics (Taylor et al., 2004; Vinogradov et al., 2002).

In present study different constituents were screened according to their multiple use and advantages which includes effective, economic, stable and easy to availability as well as in formulation. We have used PLGA (Poly (D, L- Lactide co glycolide) as a biodegradable polymer, mPEG (Methoxy Poly ethylene glycol) as a cross linker to the polymer (PLGA) and Cisplatin as an anticancer drug. All ingredient contains different functionalities in each which are: 1. PLGA, as it is a FDA approved, less immunogenic, biodegradable (biodegrades in its two monomer i.e. PLA (poly Lactic acid) and PGA (poly Glycolic acid), which are the end products of different metabolic pathways in the body, so body can handle it in its natural way) and biocompatible, and having ability to encapsulate both hydrophobic as well as hydrophilic drugs in a great extent (Song et al., 2008). 2. mPEG [Methoxy Poly ethylene glycol] as it avoids opsonization of the nanoparticles from the RES (stealth, hindering effect and shading and shielding effect) (Vlerken et al., 2007), it also gives stability to the formulation (Mao et al., 2006), it increases the circulation time of nanocarriers in the blood (Mao et al., 2006; Veronese and Pasut, 2005; Kommareddy et al., 2005; Hamidi et al., 2006; Gref et al., 1994), it shields to the external groups (Agrawal et al., 2007; Gupta et al., 2006; Bhadra et al., 2005; Malik et al, 2000; Satija et al., 2007) present in the particles, thus it converts it into less toxic moiety (Brigger et al., 2004). PEG can be used as a P-gp inhibitor, thus effective in MDR associated cancer cells treatment (Schinkel and Jonker, 2003; Choi and Shin, 2005). 3. Cisplatin as a potent anticancer drug, it was reported that Cisplatin has showed highest antitumor effect thus we used Cisplatin as a model drug for our study (Li et al., 2008; Hogberg et al., 2001; Hundahl, 2002).

The overall goal of the present study therefore was to design novel PEGylated nanoparticles (PEGyNPs) which have all the functionalities in a single stable construct, and compare their effects with non PEGylated nanoparticles.

METHODS

Materials

PLGA (MW 8000 Da, Copolymer ratio 50:50) was obtained as a gift sample from Purac Biotach, Holland, Cisplatin was obtained as a gift sample from Khandelwal laboratories, Mumbai, India, stannous octoate, Methoxy polyethylene glycol (mPEG) 2000 (MW 2000 Daltons) was purchased from sigma aldrich, India. Dialysis membrane (MWCO 5 and 10 KD) were purchased from Himedia labs, India. All other chemicals were used of extra pure grade.

Method

I) Synthesis of mPEG-PLGA copolymer

The mPEG-PLGA copolymers were synthesized by ring opening polymerization under vacuum using stannous octoate as catalyst according to Beletsi et al., 2005, briefly, PLGA and mPEG (ratio 5:45) were introduced into a bottle-neck flask. Stannous octoate (0.5%, by mass), was dissolved in hexane and added to the reaction mixture. The feed was degassed through vacuum / nitrogen cycles and applied to the molten mixture at 135°C. The flask was sealed under vacuum. Then, the polymerization reaction was carried out at 180°C for 5h under vacuum. The synthesized copolymer was recovered by dissolving in dichloromethane followed by precipitation in ice-cold diethyl ether thrice. The precipitated copolymer was filtered out (MWCO 10000) and dried under vacuum at 40°C for 24h. Synthesis was confirmed by ^{1}H NMR spectroscopy.

Preparation of PEGyNPs and PLGANPs formulation (solvent evaporation method)

PEGylated nanoparticles (PEGyNPs) or PLGA nanoparticles were prepared by the method described by Patil et al., 2008 and Song et al., 2008, with slight modification. Briefly, polymer (mPEG-PLGA copolymer for PEGyNPS & PLGA polymer for PLGA NPs) (20 mg) was dissolved in 1.30 ml of DCM, and 0.65 ml of acetone, drug solution (cisplatin solution in DMSO) was also added to it. An oil-in-water (o/w) emulsion was formed by injecting the polymer solution in 20 ml of polyvinyl alcohol (PVA) (1%w/v) solution then this solution was ultrasonicated using probe sonicator (Soniweld, Mumbai, India) for 2.00 min over an

ice bath at 50 W output over an ice bath. Then this was placed for stirring in magnetic stirrer and stirring was continued for 6 h for complete removal of organic solvents. The formed nanoparticles were recovered by ultracentrifugation (148,000g for 35 min in 4^0C). After recovery they were dialyzed thrice with deionized distilled water to dilute the micelles formed due to self assembly of mPEG-PLGA block copolymer, and finally lyophilized.

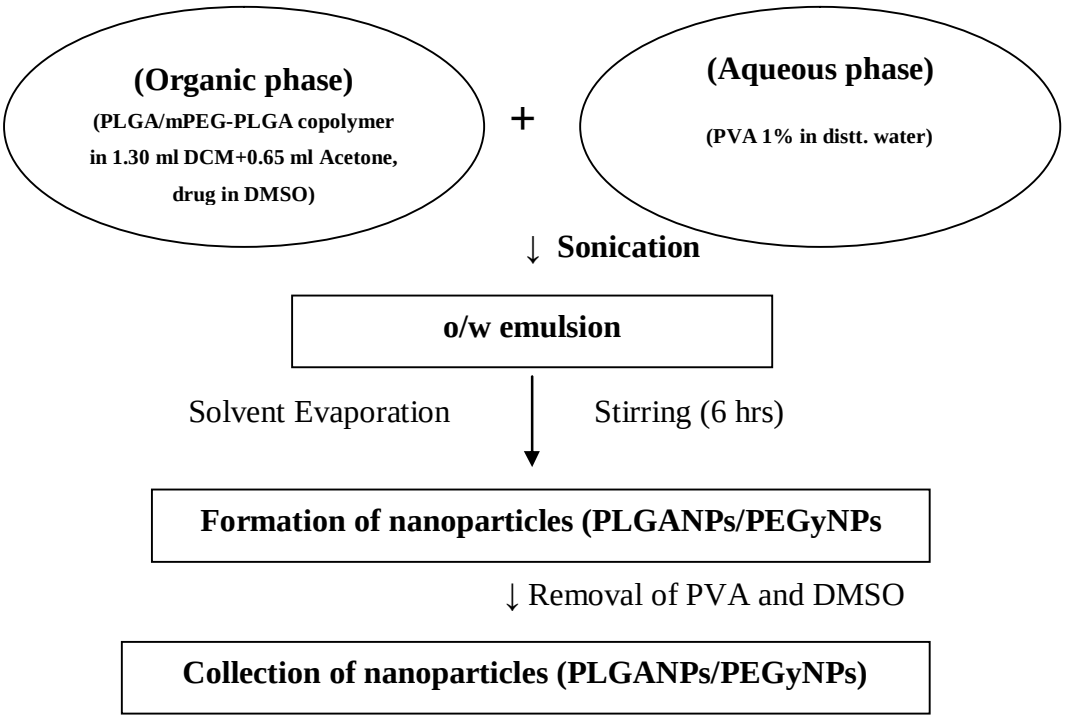

Fig.1: Schematic diagram depicting method of preparation of nanoparticles (PLGANPs/PEGyNPs).

Particle size analysis and Zeta potential

Particle size and size distribution was determined by photon correlation spectroscopy using a Zeta sizer (DTS Ver. 4.10, Malvern Instruments, England). A dilute suspension of NPs (20µg/ml) was prepared in deionized distilled water and measurements were taken in specific disposable cuvettes and recorded. Whereas zeta potential of PLGANPs and PEGyNPs were recorded in same dilution in 1 mM/L NaCl solution and then analyzed in Zetasizer (DTS Ver. 4.10, Malvern Instruments, England).

Surface Morphology (SEM)

The samples for SEM were prepared by lightly sprinkling the NPs powder on a double adhesive tape, which was stuck on an aluminum stub. The stubs were then coated with gold to a thickness of about 300 Å using a sputter coater. All samples were examined under a scanning electron microscope (LEO 435 VP, Eindhoven, Netherland) at an acceleration voltage of 30 kV, and photomicrographs were taken.

Particle Morphology (TEM)

Transmission electron microscope was used as a visualizing aid for particle morphology. The sample (10µL) was placed on the grids and allowed to stand at room temperature for 90 sec. Excess of fluid was removed by touching the edge with filter paper. Samples were examined after negative staining with phosphotungstanic acid under a transmission electron microscope (Philips Morgangni 268, Eindhoven, Netherland) at an acceleration voltage of 20 kV and images were taken.

Entrapment efficiency

Entrapment efficiency of the prepared system was determined using the procedure given by Song et al., 2008, briefly cisplatin loaded PLGANPs/ PEGyNPs were taken and ultracentrifugation was carried out at 148,000g at 4°C for 35 min. Then the supernatant was

removed and kept it aside for sedimentation of nanoparticles, sediments were washed twice with 2% PVA solution for removal of adsorb drugs. The washing solution (2% PVA) was further removed by ultracentrifugation, as described above. To dissolve nanoparticles completely DMSO was added on it. 2 ml of this dissolved solution was taken and further mixed with 1 ml of 4 M HCl–and 1 ml of 0.4 M $SnCl_2$ solution to develop chromophores on it as described by Cafaggi et al., 2007. Then cisplatin content in the solution was determined spectrophotometrically at 398 nm by taking 1 ml of 4 M HCl–and 1 ml of 0.4 M $SnCl_2$ solution, DMSO and PLGANPs/ PEGyNPs as a blank.

In vitro drug release study

In vitro drug release study is a prerequisite for evaluating the *in vivo* performance of a drug delivery system because the *in vitro* drug release profile provides the most sensitive and reliable information for *in vivo* evaluation that helps in ascertaining the future behavior of the designed formulation with regard to its drug release pattern and the time duration of its action in a biological system.

The *in vitro* cisplatin release was investigated at room temperature using PBS (pH 7.4) as dialysis media. 10 ml of drug-loaded PLGANPs/ PEGyNPs were placed in a dialysis bag (MWCO 5,000 Da.) and this was dialyzed against 100 ml of PBS (pH 7.4). At predefined intervals, 1ml of receiving buffer solution was withdrawn and cisplatin content was determined spectrophotometrically at 398 nm after addition of $SnCl_2$ - HCl mixture Cafaggi et al., 2007. At each withdrawal the dialysis medium was replaced with 1ml of fresh medium.

RESULTS & DISCUSSION

Nanoparticle characteristics

PLGANPs and PEGyNPs were prepared by the solvent evaporation method described by Patil et al. 2009 and Song et al. 2008. Before that the synthesis of copolymer (mPEG-PLGA for PEGyNPs) was synthesized and the final product of copolymer confirmed by ^{1}H NMR spectroscopy results. The ^{1}H NMR spectrum (Fig-2) shows proton peaks at 3.5 ppm for NH_2 and NH, and ~1.0 ppm for CH_2 which confirms presence of PEG, and hydrogen of the methine group of the lactic acid unit of the PLGA copolymer resonated at 5.3 ppm, whereas those of the methylene group of the glycolic acid unit appeared at 4.8 ppm and ~1.5 ppm of CH_3 confirms presence of PLGA. The peaks shown in the Fig. 2, also matched with the peaks of PEG and PLGA as described by Yadav et al., 2007; Zhao and Yung 2008 and Patil et al., 2008. So it can be concluded that the synthesis of copolymer was successful.

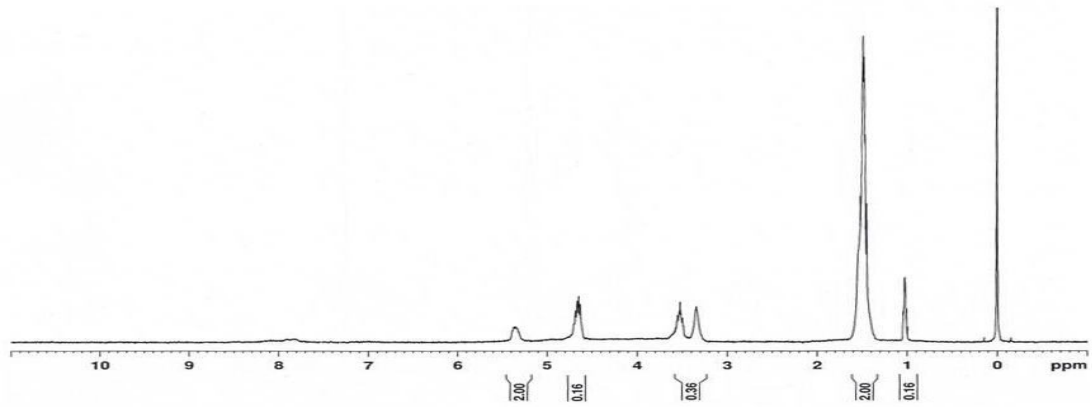

Fig. 2: ^{1}H NMR Spectrum of mPEG-PLGA copolymer

The PEGylated nanoparticles (PEGyNPs) were prepared using the synthesized copolymer of mPEG-PLGA. These prepared PEGyNPs and PLGANPs were characterized for shape and surface morphology, particle size, drug entrapment efficiency and, for Zeta potential. The PLGANPs were obtained in an average size of 170±6.5 nm with an entrapment efficiency of 74.9±2.3 % where as PEGyNPs were found to be in an average size range of 186±4.2 nm with an entrapment efficiency of 76.9±3.1%. The Zeta potential of PLGANPs was more negative (-19.43±1.5) as compared to PEGylated nanoparticles (-

7.87±1.1) might be due to presence of free –COOH group in PLGA polymer, whereas in PEGyNPs complete coating of PEG resulted decrease in Zeta potential.

The SEM and TEM photomicrograph exhibits that NPs (PLGANPs or PEGyNPs) are spherical in shape and PEG chains orientation is clear on them (Fig 3 and 4). The average size of both NPs was found to be less than 200 nm (Fig. 3A, 4A and 3B, 4B). Results indicating that optimized formulation of PLGA nanoparticles initially confirms burst release but optimized formulation of PEGyNPs overcomes burst release might be due to complete hiding of core through PEG coating in the surface. Later on drug release was found to be 22.18±1.4 % after 72 h in case of PLGANPs, while PEGylated nanoparticles exhibited 20.41±1.1 % drug release in 72 h. which is less than PLGA nanoparticles (Fig. 5). This could also be due to complete hindrance of core through PEG over the surface of NPs. This more sustained release of drug from PEGyNPs also increases the effectiveness of carrier for necrosis of tumor vasculature.

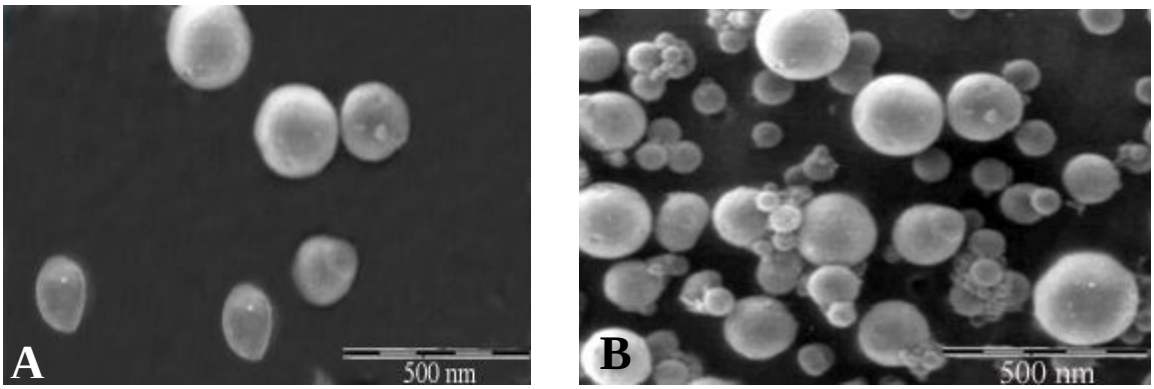

Fig. 3: SEM photomicrograph of (A) PLGANPs and (B) PEGyNPs

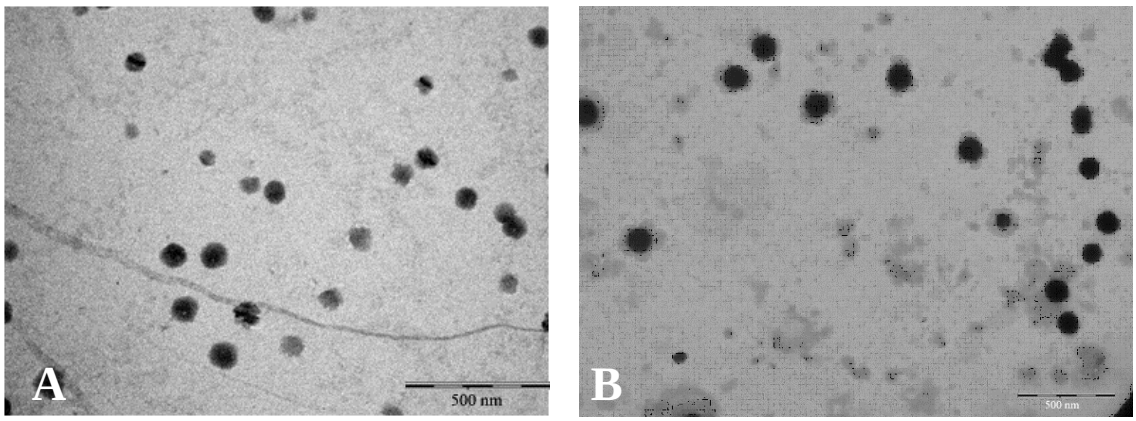

Fig. 4: TEM photomicrograph of (A) PLGANPs and (B) PEGyNPs

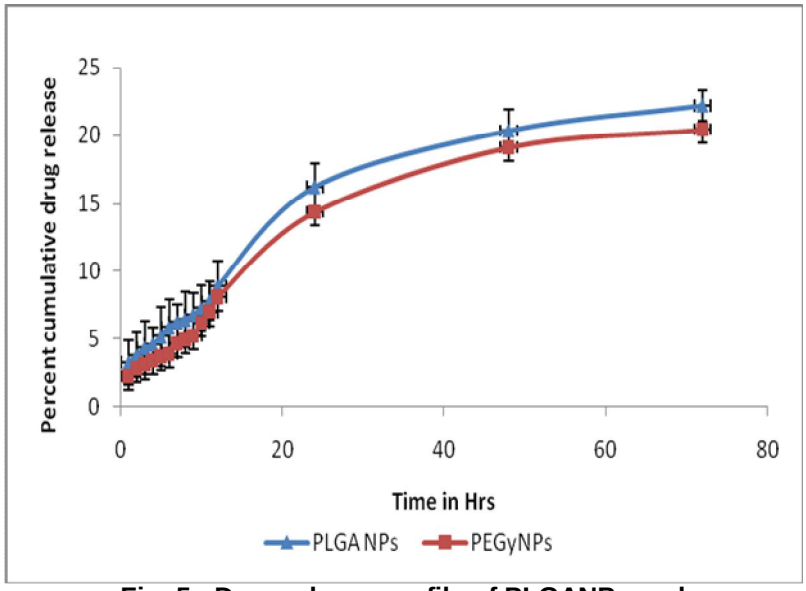

Fig. 5: Drug release profile of PLGANPs and PEGyNPs in PBS (pH 7.4).
S.D.= ± 3

CONCLUSION

In this paper, the copolymerization method was used to synthesize block copolymer. The optimization was aimed to incorporate water soluble drug in the single step O/W method instead double emulsification method W/O/W. The PEGyNPs enables more entrapment of water soluble drugs and more sustained release of nanoparticles as compared to PLGA nanoparticles. This systematic investigation reported here might promote the development of PEGylated nanoparticles and can be targeted in place of mono functional nanoparticles.

ACKNOWLEDGEMENTS

Mr. Anand Mahalwar acknowledges Suresh Gyan Vihar University, Jaipur, Rajasthan for proving facilities to conduct the work. Author also wants to thanks AIIMS for providing SEM & TEM facility.

REFERENCES

1. Bareford LM and Swaan WP. Endocytic mechanisms for targeted drug delivery. Adv Drug Del Rev. 2007;59:748–758.
2. Lee SE, Gao Z and Bae YH. Recent progress in tumor pH targeting nanotechnology. J of Cont Rel. 2008.
3. Fojo T and Coley HM. The role of efflux pumps in drug-resistant metastatic breast cancer: new insights and treatment strategies. Clin Breast Cancer. 2007;7:749–756.
4. O'Connor R. The pharmacology of cancer resistance, Anticancer Res. 2007;27:1267–1272.
5. Mohajer G, Lee ES and Bae YH. Enhanced Intercellular Retention Activity of Novel pH-sensitive Polymeric Micelles in Wild and Multidrug Resistant MCF-7 Cells. Pharm Research. 2007;24:9.
6. Sehested M, Skovsgarrd T, van Deurs B and Winther-Nielsen H. Increase in nonspecific adsorptive endocytosis in anthracycline- and vinca alkaloid-resistant Ehrlich ascites tumor cell lines. J Natl Cancer Inst. 1987;78:171–179.
7. Simon SM and Schindler M. Cell biological mechanisms of multidrug resistance in tumors. Proc Natl Acad Sci. 1994; 91:3497– 3504.
8. Cataldo AM, Petanceska S, Peterhoff CM, Terio NB, Epstein CJ, Villar A, Carlson EJ, Staufenbiel M and Nixon RA. App gene dosage modulates endosomal abnormalities of Alzheimer_s disease in a segmental trisomy 16 mouse model of down syndrome. J Neurosci. 2003;23:6788–6792.
9. Lazzarino DA, Blier P, Mellman I. The monomeric guanosine triphosphatase rab4 controls an essential step on the pathway of receptor-mediated antigen processing in B cells. J Exp Med. 1998;188:1769–1774.

10. Schinkel AH and Jonker JW. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. Adv Drug Delivery Rev. 2003;55:3-29.
11. Choi JS and Shin SC. Enhanced paclitaxel bioavailability after oral coadministration of paclitaxel prodrug with niringin to rats. Int J Pharm, 2005;292:149-156.
12. Kabanov AV, Batrakova EV and Miller DW. Pluronic block copolymer as modulators of drug efflux activity in the Blood-Brain barrier. Adv Drug Del Rev. 2003;55:151-164.
13. Labhasetwar V. Nanoparticles for drug delivery. Pharm news. 1997;4:28-31.
14. Taylor S, Qu LW, Kitaygorodskiy A, Teske J, Latour RA and Sun YP. Synthesis and characterization of peptide functionalized polymeric nanoparticles. Biomacromolecules. 2004;5(1):245–248.
15. Vinogradov SV, Bronich TK and Kabanov AV. Nanosized cationic hydrogels for drug delivery: preparation, properties and interactions with cells. Adv Drug Deliv Rev. 2002; 54(1):135–147.
16. Song X, ZhaoY, Hou S, Xu F, Zhao R, He J, Cai Z, Li Y and Chen Q. Dual agents loaded PLGA nanoparticles: Systematic study of particle size and drug entrapment efficiency. Eur J Pharm and Biopharm. 2008;69:445–453.
17. Vlerken LE, Vyas TK and Amiji MM. Poly(ethylene glycol)-modified Nanocarriers for Tumor-targeted and Intracellular Delivery. Pharmaceutical Research. 2007;24: 8.
18. Mao S, Neu M, Germershaus O, Merkel O, Sitterberg J and Bakowsky U. Influence of polyethylene glycol chain length on the physicochemical and biological properties of poly(ethyleneimine)-graft-poly(ethylene glycol) block copolymer/SiRNA polyplexes. Bioconjug Chem. 2006;17:1209–1218.
19. Veronese FM and Pasut G. PEGylation, successful approach to drug delivery. Drug Discov Today. 2005;10:1451–1458.
20. Kommareddy S, Tiwari SB and Amiji MM. Long-circulating polymeric nanovectors for tumor-selective gene delivery. Technol Cancer Res Treat. 2005;4:615–625.
21. Hamidi M, Azadi A and Rafiei P. Pharmacokinetic consequences of pegylation. Drug Deliv. 2006; (13) 399–409.
22. Gref R, Minamitake Y, Peracchia MT, Trubetskoy V, Torchilin V and Langer R. Biodegradable long-circulating polymeric nanospheres. Science. 1994;263:1600–1603.
23. Agrawal P, Gupta U and Jain NK. Glycoconjugated peptide dendrimers based nanoparticulate system for the delivery of chloroquine phosphate. Biomaterials 2007; 28:3349–59.
24. Gupta U, Agashe HB, Asthana A and Jain NK. Dendrimers: novel polymeric nanoarchitectures for solubility enhancement. Biomacromolecules. 2006;7:649–58.
25. Bhadra D, Yadav AK, Bhadra S and Jain NK. Glycodendrimeric nanoparticulate carriers of primaquine phosphate for liver targeting. Int J Pharm. 2005;295:221–33.
26. Malik N, Wiwattanapatapee R, Klopsch R, Lorenz K, Frey H, Weener JW, Meijer EW, Paulus W and Duncan R. Dendrimers: relationship between structure and biocompatibility in vitro and preliminary studies on the biodistribution of 125I labeled polyamodoamine dendrimers in vivo. J Controlled Release. 2000;65:133–48.
27. Satija J, Gupta U and Jain NK. Pharmaceutical and biomedical potential of surface engineered dendrimers. Crit Rev Ther Drug Carrier Syst. 2007;24(3):261–315.
28. Zhao H and Yung LYL. Selectivity of folate conjugated polymer micelles against different tumor cells. International Journal of Pharmaceutics. 2008;349:256–268.
29. Li X, Li R, Qian X, Ding Y, Tu Y, Guo R, Hub Y, Jiang X, Guo W and Liu B. Superior antitumor efficiency of cisplatin-loaded nanoparticles by intratumoral delivery with decreased tumor metabolism rate. European Journal of Pharmaceutics and Biopharmaceutics. 2008;70:726–734
30. Hogberg T, Glimelius B and Nygren P. SBU-group. Swedish Council of Technology Assessment in Health Care, a systematic overview of chemotherapy effects in ovarian cancer, Acta Oncol. 2001; 40:340–360.

31. Hundahl SA. Surgical quality in trials of adjuvant cancer therapy. J Surg Oncol. 2002;80: 177–180.
32. Patil YB, Toti US, Khdair A, Mac L, Panyamc J. Single-step surface functionalization of polymeric nanoparticles for targeted drug delivery. Biomaterials. 2008;20: 1–8.
33. Cafaggi S, Russo E, Stefani R, Leardi R, Caviglioli G, Parodi B, Bignardi G, Totero DDe, Aiello C and Viale M. Preparation and evaluation of nanoparticles made of chitosan or N-trimethyl chitosan and a cisplatin–alginate complex, J of Controlled Release. 2007;121:110–123
34. Yadav AK, Mishra P, Mishra AK, Mishra P, Jain S and Agrawal GP. Development and characterization of hyaluronic acid–anchored PLGA nanoparticulate carriers of doxorubicin. Nanomedicine: Nanotechnology, Biology, and Medicine. 2007;3:246–257.
35. Beletsi A, Panagi Z and Avgoustakis K. Biodistribution properties of nanoparticles based on mixtures of PLGA with PLGA-PEG diblock copolymers. Int J Pharm. 2005; 298:233-4