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EVALUATION OF RECEPTOR MEDIATED ENDOCYTOSIS ON CELLULAR INTERNALIZATION: A COMPARATIVE STUDY OF PEGYLATED NANO PARTICLES AND FOLATE ANCHORED PEGYLATED NANO PARTICLES ON MDA-MB-231 CELLS

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ABSTRACT
The present work was aimed to develop, explore & compare the use of Folate anchored PEGylated nanoparticles (FANPs) made of the copolymer poly (lactide-co-glycolide) (PLGA) - polyethylene glycol (PEG) & - folic acid with non-anchored PEGylated PLGA nanoparticles (PEGyNPs) for targeting solid tumor. For that first optimum cytotoxic concentration of PLGA (polymer) and cisplatin (drug) were optimized through MTT assay. The optimum size and percent entrapment efficiency were found to be 171±5.5 nm and 75.9±2.6% for PEGyNPs and 182±4.2 nm and 75.9±2.1% for FANPs. The in vitro cytotoxic activity of FANPs and PEGyNPs were investigated & compared with drug solution (cisplatin) on MDA-MB-231 breast cancer cells, which revealed that FANPs are more cytotoxic in a time dependent manner. The rhodamine B isothiocyanate loaded PEGyNPs and FANPs were prepared & compared for cell uptake studies which confirmed that targeted NPs (FANPs) were more taken up by the MDA-MB-231 cells. To determine the effect of ligand (folic acid) on internalization, cells were incubated with FANPs and PEGyNPs. Results confirmed that the presence of ligand gradually increases internalization of carriers and exhibited maximum uptake of FANPs than PEGyNPs. Results suggesting that FANPs are promising approach for targeting solid tumor & to achieve deeper cellular internalization.

Key words: PLGA poly (lactide-co-glycolide), Nanoparticles, Folate anchored nanoparticles, Cytotoxicity, Cell uptake.

INTRODUCTION
Cisplatin (cis-diaminedichloroplatinum) is one of the most frequently used antineoplastic agent (Wittgen et al., 2007), as it has proved its superior antumor activity against variety of solid tumors and used in most of first-line chemotherapeutic (Wittgen et al., 2007; Li et al., 2008; Hsi et al., 2001; Michail 2002). Platinum has a strong affinity to bind at N7 of guanine on DNA. The binding to DNA results in an altered protein conformation and changes in biological activity, especially when enzymatic reactions are affected (Jameson and Lidyard 1999; Jakubec et al., 2003; Wouw and Gandolomence 1999). It shows high toxicity i.e. nephrotoxicity, gastrointestinal toxicity, ototoxicity, cardiotoxicity and neurotoxicity (Reedijk 1999; Bugarcic et al., 2008) when it is given in non-site specific manner, thus it limits its therapeutic potential.

To make it potent, past research was aimed to reduce its toxicity by making either its complex with
different polymers like polycarboxylates (Arichizer 

letters et al., 1999), poly(acrylamides) (Ferrani et al., 1999), polyamidoamine dendrimers (Malik et al., 1999) and the complexes with N-(2-hydroxypropyl) methacrylamide (Gianazi et al., 1999), or encapsulating it in the form of vesicular systems, such as liposomes (Meerum Terwogt et al., 2002; Vesel et al., 2001), FEGylated liposomes (Gewman et al., 1999), poly(aspartic acid–poly(ethylene glycol) micelles (Nishiyama et al., 2002) and poly(caprolactone)-poly(ethylene glycol) or poly(caprolactone)-poly[2-(N,N-dimethylamino) ethyl methacrylate] micelles (Xu et al., 2006). Some other approaches also utilized by the researchers to reduce its side effects such as encapsulation of it in the polymeric matrix, such as PLGA microparticles, PLGA nanoparticles (Moreno et al., 2008), FEGylated PLGA nanoparticles (Gryparis et al., 2007), FEGylated FCL nanoparticles (Li et al., 2008) as cisplatin with long-circulating carriers (PEGylated nanoparticles) drug pharmacokinetics and results in increased drug accumulation in tumors, based on the “enhanced permeability and retention” (EPR) effect (Kwon et al., 2001; Gryparis et al., 2007) the FEGylated PLGA nanoparticles (Gryparis et al., 2007), FEGylated FCL nanoparticles was formulated (Li et al., 2008).

To the best of our knowledge, no studies so far have been reported on, ligand anchored targeted delivery of encapsulated cisplatin in nanocarriers for cancer cells, hence, a target specific, stealth polymeric nanocarrier (FANPs) where designed & compared with another polymeric FEGylated nanocarrier (FEGYNPs) having also ability to target [passive targeting due to enhanced permeability and retention (EPR) effect (Maeda, 2000)] as it was reported earlier that folate acid internalizes via caveolae assisted receptor mediated endocytosis, and thus it makes it efficient to release on the nucleus of the cytosolic environment (Kim et al., 2007; Zhao and Yang 2005; Lemmon and Low 1991; Bennis et al., 2001; Shimoda et al., 1998; Ke et al., 2003; Zhou et al., 2002; Li et al., 1998) with bypass mechanism of harsh environment of lysosomal degradation, where the nucleus resides (Chang et al., 1992; Dasaty et al., 2002; Schmittke 2001; Murthy et al., 2003; Bathra et al., 2004). So that folate acid is used as ligand for present investigation.

Folate anchored nanoparticles consist specific constituents i.e., 1. Folic acid- (i) makes carrier target potential (Kim et al., 2007; Konda et al., 2000) (ii) provides deeper cellular internalization & nucleus directed release due to caveolin assisted receptor mediated endocytosis (Murthy et al., 2003), (iii) due to strong uptake of folic acid at cellular nucleus (Kim et al., 2007) can manage efflux mechanism of P-glycoprotein (P-gp) causes multidrug resistance (MDR) (Sharon, 1997), 2. PEG- (i) makes carriers long circulatory by preventing opsonization (Owens DE and Peppas NA, 2006; Vlasken et al., 2007) (ii) confers hydrophilic layer over the surface of carrier thus MDR receptors (hydrophobic vacuum cleaner) will unable to efflux them out (Sharon, 1997), (iii) can improve encapsulation efficiency of hydrophobic drug and also increases sustainability in drug release (Gref et al., 1995; Peracchia et al., 1997; Avgoustakis et al., 2002), 3. PLGA-(i) biocompatible and biodegradable polymer (Fu et al., 2002; Claudia S and Erc D, 1998), (ii) improves encapsulation efficiency of hydrophilic drug (Lee et al., 2002; Song et al., 2008), (iii) sustained as well as controlled release can be possible (Fu et al., 2002; Lee et al., 2002), which makes it potent for targeting and achieving maximum cytotoxicity in the neoplasm. So, in this present study the targeting potential as well as importance of ligand (folic acid) on cellular internalization were evaluated & compared with another passive targeted FEGylated nanoparticles.

MATERIAL & METHODS

Materials

PLGA (MW 8000 Da, Copolymer ratio 50:50) was obtained as a gift sample from Purac Biotech, Holland, Bis amine PEG (NH2-PEG-NH2) (MW 4000 Da) was obtained as a gift sample from Huntsman Pvt. Ltd, USA. Cisplatin was obtained as a gift sample from Khandelwal laboratories, Mumbai, (India), Dialysis membrane (MWCO 5, 10, 12 and 14 KD) were purchased from Across labs, (India). Breast cancer cell line MDA-MB-231 was purchased from National Center for Cell Science, Pune, (India), Stannous octoate, Methoxy polyethylene glycol (mPEG) 2000 (MW 2000 Daltons), Folic acid, MTT (3-(4, 5-dimethylthiazol-2)-2, 5-diphenyltetrazolium bromide), Culture medium RPMI 1640, Antibiotic and antymycotic solution, and L-glutamine solution were purchased from Sigma Aldrich, India. Fetal Calf Serum was purchased from Hyclone, USA. All other chemicals were used of extra pure grade.

Cell culture

The human breast cancer cell line MDA-MB-231 was grown routinely in RPMI 1640 medium supplemented with 10% FBS (fetal calf serum), 1% Antibiotic antymycotic solution, and 1% L-glutamine. Cell lines were maintained in humidified 5% CO2 incubator at 37°C till confluent of cells.

Methods

1) Synthesis of PLGA-PEG- Folate conjugate

The PLGA-PEG-Folate conjugate was synthesized in four steps i.e., Folic acid activation, PLGA activation synthesis of Folate-PEG-NH2, and final synthesis of PLGA-PEG-Folate conjugate as depicted in scheme 1.

Activation of Folic Acid
Folic acid was activated according to the method described by Patil et al., 2009, with slight modification briefly; folic acid (2.0 gms) was dissolved in a mixture of anhydrous dimethyl sulfoxide (DMSO 80 ml) and triethylamine (TEA 1.0 ml), and this was stirred overnight at dark. The resultant solution was further stirred for 18 hrs, after mixing of dicyclohexylcarbodiimide (DCC 1.0 gms) and N-hydroxysuccinimide (NHS, 0.56 gms) in it. The side product dicyclohexylureas (DCU) was removed by filtration. DMSO and TEA were evaporated under vacuum.

**Activation of PLGA Polymer**

Activation of PLGA (average molecular weight of 8000 Da) was carried out according to the method described by Zhao and Yung, 2008 briefly; 2 gms of PLGA was activated by DCC (36.5 mg) and NHS (31.5 mg) (molar ratio 1:1:1.1, respectively) in 5 ml of dichloromethane (DCM) at room temperature under nitrogen gas for 24 h. The resultant was filtered to remove product dicyclohexylureas (DCU). After filtration, activated PLGA was precipitated by ice-cold diethyl ether and dried under vacuum.

**Preparation of Folic Acid-PEG-NH$_2$**

Folic acid was linked to the bis amine PEG (NH$_2$-PEG-NH$_2$) according to the method described by Patil et al., 2009, with slight modification, briefly; Bis-amine-PEG 2.0 gms (150 times more than NH$_2$-Folic acid), average molecular weight of 4000 Da) was dissolved in acetonitrile (4.0 ml). Methylene chloride (2.0 ml) and TEA (1.6 ml) were added on it and the solution was stirred for 1 min. NHS- Folic acid (500 mg) was introduced on the above mixture and stirred overnight under nitrogen. The reaction was stopped by the slow addition of diethyl ether (10-15 ml) to precipitate the polymer and to separate the unreacted PEG. Then precipitated polymer was filtered and washed with diethyl ether. The unreacted polymers were removed by dialysis (dialysis membrane MWCO, 4000). The dialyzed product was lyophilized and then analyzed for folic acid conjugation through $^1$H NMR spectroscopy.

**Synthesis of PLGA-PEG-folic acid conjugate copolymer**

The final synthesis was carried out according to the method described by Zhao and Yung, 2008, with slight modification. Activated PLGA 1.0 gms and 1.2 gms of Folic acid-PEG-NH$_2$ was added to 5 ml of DMSO at room temperature for 8 hrs under nitrogen gas. The final product was precipitated in ice cold diethyl ether, and then this was further dissolved in DMSO and dialyzed for the removal of DMSO and unreacted polymers against deionized water over 72 hrs (MWCO 12000). The further collected copolymer (Folate-PEG-PLGA) was dialyzed in DMSO (MWCO 14000) to remove the PLGA-PEG-PLGA copolymers. Then, the dialyzed copolymers were recovered by dialysis against deionized water over 72 hrs, and also from vacuum dryer. Finally these were lyophilized, and final product was analyzed for conjugation through NMR spectrum. The conjugate was dissolved in CDCl$_3$, and $^1$H NMR spectra was recorded with employing NMR Spectrometer (Bruker DRX-300 Germany).

**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, PLA 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 19000) and dried under vacuum at 40°C for 24h. Synthesis was confirmed by $^1$H NMR spectroscopy.

**Preparation of PEGyNPs and FANPs formulation (solvent evaporation method)**

Folic acid-PEG-PLGA nanoparticles (FANPs) or PEGyNPs were prepared by the method described by Patil et al., 2009 and Song et al., 2008, with slight modification briefly, polymer (to make FANPs, PLGA-PEG-folate conjugate & for PEGyNPs, mPEG-PLGA copolymer) (20 mg) was dissolved in 1.50 ml of DCM, and 0.65 ml of acetone, drug solution (cisplatin soluble in DMSO) was also added on 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) at 50 W for 2.0 min over an ice bath. Then this was placed for stirring on a magnetic stirrer and stirring was continued for 5 h for complete removal of organic solvents. The formed nanoparticles were recovered by ultracentrifugation (148,000g for 35 min in 4°C). After recovery, they were dialyzed thrice with deionized distilled water to dilute the micelles formed due to self-assembly of PLGA-PEG block copolymer, and finally lyophilized.

**Particle size analysis and Zeta potential**

Particle size and size distribution was determined by photon correlation spectroscopy using a Zetasizer (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 PEGyNPs and FANPs 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 100 Å using a sputter coater. All samples were examined under a scanning electron microscope (LEO 435 VP, Eindhoven, Netherlands) 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 (10 μL) was placed on the grid 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 phosphotungstic acid under a transmission electron microscope (Philips Morgagni 268, Eindhoven, Netherlands) 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 FANPs / 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 SnCl₂ solution to develop chromatophores on it as described by Cagliari 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 SnCl₂ solution, DMSO and PLGA-PEG-Folic acid (FANPs)/ 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 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 FANPs / 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, 1 ml of receiving buffer solution was withdrawn and cisplatin content was determined spectrophotometrically at 398 nm after addition of SnCl₂ - HCl mixture Cagliari et al., 2007. At each withdrawal the dialysis medium was replaced with 1 ml of fresh medium.

In vitro Cytotoxicity study

The cytotoxicity of drug (Cisplatin) and blank PLGA polymer were investigated by MTT (3-(4, 5-dimethylthiazolyl-2)-2. 5-diphenyltetrazolium bromide) assay (Grypars et al., 2007; Cagliari et al., 2007). MDA-MB-231 cells were seeded in 10% fetal calf serum (FCS) containing RPMI 1640 with 1% Antibiotic antymycotic and 1% L-glutamine solution till the flask appeared to be confluent with cells. Cells were scraped with the help of scraper, pelleted down and cell number was counted by Trypan Blue exclusion method in Neubaur’s chamber under 10X magnification. Cells were suspended in RPMI 1640 medium supplemented with 10 % heat inactivated fetal calf serum (FCS) and antibiotic antymycotic solution and L-Glutamine, cells dilution was kept 2x10⁶/ml. 100 μl of cell suspension was added to each well of the 96 well tissue culture plates. Cells were stimulated with various concentrations of test reagent, unstimulated cells were taken as control. Complete volume of well was made 200 μl by adding 5% FCS supplemented medium (RPMI 1640). Culture plate was incubated at 37°C in 5% CO₂ humidified incubator for three days.

Cytotoxicity of drug and polymers were measured by adding 20 μl MTT dye (5mg/ml in PBS, pH 7.4) per well of 96 well culture plate. Plate was further incubated for 3 h at 37°C in a humidified chamber containing 5% CO₂. The Formazan crystals formed due to reduction of dye by viable cells. Each well was dissolved in 150 μl DMSO (Di-methyl sulfoxide) and optical density (OD) was read at 490 nM by SpectraMax M2 plate reader (Molecular devices, USA). The experiment was performed at triplicate and repeated for three times for accuracy. Cytotoxicity was expressed as percentage reduction in cell viability, which was calculated from the ratio between the number of cells treated with the different test formulations (drug and polymer concentrations) and that of non-treated cells (control).

The optimum cytotoxic concentration of drug and polymer was recorded and expressed in graph. After getting the optimized concentration of drug and polymer we used the same optimum concentration of drug and polymer to prepare PEGyNPs and FANPs then again checked cytotoxic potential of both formulations.

Cell uptake study by MDA-MB-231 cells

PEGyNPs loaded with rhodamine B isothiocyanate (dye) and, FANPs loaded with dye (rhodamine B isothiocyanate) were prepared to investigate their uptake by MDA-MB-231 cancer cells. The dye loaded NPs were prepared by the same method as was used to prepare drug.
.loaded NPs, only drug was replaced with dye which was mixed in the PVA solution. The dye loaded PEGyNPs and FANPs were purified from unloaded dye by centrifugation. 1 ml of dye loaded FANPs and PEGyNPs suspensions were taken and volume was made up by 1 ml of complete RPMI 1640 solution and added to monolayer’s of MDA-MB-231 breast cancer cells (1.5x10^4) which were grown in 24 wells culture plate and incubated for 24, 48 and 72 hours at 37°C. After suitable time period (24, 48 or 72 hrs), these were pelleted down through centrifugation (300 g for 5 min in room temperature). Then, the supernatant was discarded and volume was made up by staining buffer [PBS pH 7.4, Sodium Azide (1N), BSA] up to 2 ml. Fluorescence of the dye was measured by acquisition of cells on fluorescence activated cell sorting analyzer (FACS) instrument [Ania (Becton Dickinson Biosciences, USA)]. The data of time kinetics of fluorescence related cell uptake was analyzed by using BD FACS Diva software.

RESULTS & DISCUSSION
Nanoparticle characteristics
FANPs and PEGyNPs were prepared by the solvent evaporation method described by Patil et al. 2009 and Song et al. 2008. Before the conjugation of copolymer (PLGA-PEG-Folate for FANPs and mPEG-PLGA for PEGyNPs) was synthesized and the final product of copolymer confirmed by 1H NMR spectroscopy results. The 1H NMR spectrum (Fig-1) shows proton peaks at 3.5 ppm for NH, and NH, and -1.0 ppm for CH3 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 CH3 confirms presence of PLGA. The peaks shown in the Fig. 1, also matched with the peaks of PEG and PLGA as described by Yadav et al., 2007; Zhao and Yang 2008 and Patil et al., 2008. In Fig.2 the additional proton peaks at 6.80 and 7.21 ppm exhibit the presence of C=NH, and 1-benzene CH, respectively which confirm the presence of Folic acid-PEG, whereas other proton peaks were same as Fig. 1 which confirms the presence of PEG and PLGA. The peaks shown in the Fig. 2, also matched with the peaks of PLGA, PEG and Folate as described by Zhao and Yang 2008; and Patil et al., 2008. So it can be concluded that both the conjugation has been successfully developed.

The folate anchored PEGylated nanoparticles (FANPs) and PEGylated nanoparticles were prepared using the synthesized copolymer PLGA-PEG-Folate and mPEG-PLGA respectively. These prepared PEGyNPs and FANPs were characterized for shape and surface morphology, particle size, drug entrapment efficiency and, for Zeta potential. The PEGyNPs were obtained in an average size of 171±5.5 nm with an entrapment efficiency of 75.9±2.6%, whereas FANPs were found to be in an average size range of 185±4.2 nm with an entrapment efficiency of 75.9±2.1%. The Zeta potential of PEGyNPs was more negative (-19.8±1.5) as compared to FANPs (-6.8±1.1) might be due to presence of free –COOH group (unpolymerized with PEG) in PLGA polymer, whereas in FANPs complete coating of PEG resulted decrease in Zeta potential.

The SEM and TEM photomicrograph exhibits that NPs (FANPs 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 PEGylated nanoparticles initially confirms burst release but optimized formulation of FANPs 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 24.1±1.4 % 25.1±1.6 % after 72 h in case of PEGyNPs, while FANPs exhibited 22.4±1.1 % 23.1±1.3 % drug release in 72 h, which is less than PEGylated nanoparticles (Fig. 5). This could also be due to complete leaching of core through PEG over the surface of NPs. This more sustained release of drug from FANPs also increases the effectiveness of carrier for necrosis of tumor vasculature.

Cytotoxicity assay of drug and blank PLGA polymer
Different drug and polymer concentrations were screened and found for optimum cytotoxic effects. It was resulted that 13.5 μg/ml cisplatin concentration has showed highest cytotoxic effect than other concentrations (Fig. 6) and 720 μg/ml polymer concentration (Fig. 7) was optimum for cytotoxic effects. The results are almost similar with that reported by Gryparis et al. 2007.

In- vitro cytotoxicity of formulations (PEGyNPs and FANPs)
The in vitro cytotoxicity activity of drug loaded PEGyNPs and drug loaded FANPs (containing optimized drug and polymer concentration which were cytotoxic) on MDA-MB-231 cancer cell line was studied according to percentage reduction in cell viability (Fig. 8). The anticancer activity was compared between PEGyNPs and FANPs. Result confirms that FANPs are more cytotoxic than compared to PEGyNPs (Fig. 8) and simple drug solution (Fig. 6) might be due to receptor mediated endocytosis mechanism in the cells. FANPs internalize via caveolin assisted receptor mediated endocytosis and thus nucleus directed drug release results more cytotoxicity whereas PEGyNPs internalizes via simple endocytosis mechanism thus less cytotoxic.

Uptake of formulations by MDA-MB-231 cells
The cell uptake study of different formulations were carried out on tumor cells, MDA-MB-231 cell monolayer’s were incubated with rhodamine B
isothiocyanate loaded PEGyNPs, dye loaded FANPcs, and control, for different time intervals, i.e., 24, 48, and 72 hours at 37°C. To distinguish surface bound NPs, MDA-MB-231 cells were washed with cold PBS to remove unassociated NPs or with acidic saline to strip bound but not internalized NPs. The cells were then lysed and assayed for residual cell fluorescence using the protocol described above. Uptake of rhodamine loaded FANPs increased up to 84.45% in 72 h (Fig. 9), whereas PEGyNP uptake was found to be 65.26% in 72 h (Fig. 9), which confirms that FANPs were taken up by the cells via receptor-mediated endocytosis method and thus uptake was gradually increased as compared to PEGyNPs. Thus, it can be proved that caveolae-assisted receptor-mediated endocytosis facilitates the internalization of the nanocarriers and thus FANPs are most suitable to provide more and sustained therapeutic concentrations in the site.

Scheme 1, Synthesis of PLGA-PEG-Folate conjugate

1. Folic acid + DCC + NHS

   (NHS activated Folic acid)

2. PLGA + DCC + NHS

   NHS activated PLGA polymer

3. Preparation of folic acid-PEG-NH₂

   (NHS activated folic acid)

   Bisamine-PEG-NH₂

   Folic acid conjugated PEG polymer
4. Synthesis of PLGA-PEG-folate conjugated copolymer

![Chemical diagram]

**Scheme 1, Synthesis of NHS activated folate**

![Chemical diagram]
Fig. 1. $^1$H NMR Spectrum of mPEG-PLGA copolymer

Fig. 2. $^1$H NMR Spectrum of PLGA-PEG-Folate conjugated copolymer
Fig. 3. SEM photomicrograph of (A) PEGyNPs and (B) FANPs

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

Fig. 5. Drug release profile of PEGyNPs and FANPs in PBS (pH 7.4).
Fig. 6. MTT assay of different drug (Cisplatin) concentration

Fig. 7. MTT assay of different polymer concentration

Fig. 8. MTT assay of PEGyNPs, and FANPs

**SD = ± 3**
CONCLUSION
These studies exhibited that FANPs (folate appended PLGA nanoparticles bearing cisplatin) are more potent as compared to PEGylated nanoparticles bearing cisplatin as they are more cytotoxic and exhibited more uptake by breast carcinoma cells i.e. MDA MB-23 cells. Hence, these FANPs would be used as potent carrier for targeting the breast tumor cells and overcome the side effects of the anticancer drug and maximizes its utilization by specific tissues.

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

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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 1H 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 size, 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; O Connor, 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 (MOAT) (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 trial (Mohajer et al., 2007; Sehested et al., 1967; Simon and Schindler, 1994; Catadio 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 nanocarriers offers many benefits. These benefits include controlled drug release and protection, prolonged blood circulation times, and many other advantageous 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 Lactic 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 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 are biocompatible, and having ability to encapsulate both hydrophilic as well as hydrophobic drugs in a great extent (Song et al., 2008). 2. mPEG [Methoxy Poly ethylene glycol] as it avoids opsonisation of the nanoparticles from the RES (stealth, hindering effect and shading and shielding effect) (Vierker 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; Kommede et al., 2005; Hamidi et al., 2006; Geef et al., 1994), it shields to the external groups (Agrawal et al., 2007; Gupta et al., 2007; Ho et al., 2005; Ma et al., 2005; Satija et al., 2007) present in the particles, thus it converts it into less toxic moiety (Brieger 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 Biotech, Holland, Cisplatin was obtained as a gift sample from Khandelwar, Mumbai, India, stannous octoate, Methoxy polyethylene glycol (mPEG) 2000 (MW 2000 Da) 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 co polymers 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 reaction was continued 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 1H 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 0.85 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°C). After recovery they were dialyzed three with deionized distilled water to dilute the micelles formed due to self assembly of mPEG-PLGA block copolymer, and finally lyophilized.

\[ (\text{Organic phase}) \quad (\text{Aqueous phase}) \]
\[ 0.30 \text{ ml DCM}+0.55 \text{ ml Acetone, drug in DMSO} \quad \text{PVA 1\% in dist. water} \]
\[ \downarrow \text{Sonication} \]
\[ \text{o/w emulsion} \]
\[ \downarrow \text{Solvent Evaporation} \quad \downarrow \text{Stirring (6 hrs)} \]
\[ \text{Formation of nanoparticles (PLGANPs/PEGyNPs)} \]
\[ \downarrow \text{Removal of PVA and DMSO} \]
\[ \text{Collection of nanoparticles (PLGANPs/PEGyNPs)} \]

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 sprinking 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, Netherlands) 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 phosphotungstic acid under a transmission electron microscope (Philips Morgagni 258, Eindhoven, Netherlands) 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 disprotein 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 drug. 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 SnCl₂ 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 SnCl₂ 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 SnCl₂ - 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 ¹H NMR spectroscopy results. The ¹H NMR spectrum (Fig-2) shows proton peaks at 3.5 ppm for NH₂ and 1.0 ppm for CH₃ 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 CH₃ 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.

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, 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 75.9±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 hinderance 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
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 O/W/O. The PEGyNPs enable 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.

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