Abstract - The present investigation was aimed to develop a receptor (Transferrin) appended PEGylated PLGA long circulating nanoparticles of paclitaxel. Nanoparticles of PLGA and PEG synthesized copolymer were prepared by emulsification solvent evaporation method. The surface of PEGylated nanoparticles was modified by appending transferrin ligand for receptor mediated targeting and characterized for particle size, zeta potential, percentage drug entrapment and in vitro drug release. Cytotoxicity studies were performed on different human cancer cell lines. Confocal Laser Scanning Microscopy studies shows the internalization of the dye in the brain.

Keywords- blood brain barrier, targeting, transferrin, PEGylated Nanoparticles

I. INTRODUCTION

Brain is a delicate organ, and fruitation built very efficient ways to protect it. Unfortunately, the same mechanisms which protect it against invasive chemicals can also frustrate therapeutic interventions. Despite of enormous advances in the field, many existing pharmaceuticals are rendered ineffective in the treatment of brain associated diseases such as brain tumors, HIV encephalopathy, epilepsy, cerebrovascular diseases and neurodegenerative disorders due to our inability to effectively deliver and sustain them within the brain. (1) Treating CNS diseases is particularly challenging because a formidable obstacles i.e., the presence of the blood brain barrier (BBB) often impede drug delivery to the brain.

The function of the BBB is dynamically regulated by various cells present at the level of the BBB. By localizing drugs at their desired site of action one can reduce toxicity and increase treatment efficiency.

In response to the insufficiency in conventional delivery mechanisms, aggressive research efforts have recently focused on the development of new strategies to more effectively deliver drug molecules to the CNS. (2,3) Colloidal carriers such as polymeric nanoparticles have recently gained interest for targeting as well as sustained drug release. Nanoparticles are solid or semisolid colloidal particles ranging in size from 10 to 1000 nm with a ability to deliver a choice of drugs to various part of the body and also for sustained period of time. (4)

In the present study, emulsification solvent evaporation method was used for preparation of paclitaxel bearing plain and PEGylated nanoparticles.

II. MATERIALS AND METHOD

Materials
Paclitaxel was obtained as a gift sample from the Kandelwal Lab, Mumbai India. Poly (D-L-lactic-Co-glycolic acid) (PLGA) with L:G molar ratio of 50:50 and Mw of 20,000, Bis-PEG Mw of 10,000, Polyvinyl alcohol (PVA), human transferrin (Tf), stannous octoate, were procured from Sigma chemicals, St Louis, USA. Cellophane membrane (molecular weight cut off, 12000–14000 dalton) was procured from Himedia Ltd., Mumbai, India. All other reagents used in this study were of HPLC grade. Water wherever used was deionized and sterile water for injection.

Synthesis of PEG-PLGA copolymer conjugate
Experimentally, lactide and glycolide in a molar ratio of 4:1 and the specified amount of Bis-PEG were put in thick-walled glass tubes with the total feed weight of approx 3gm. Stannous octoate (0.03%w/w) as catalyst was dissolved in hexane and added to the feed and heated at 190°C for 2 hours. (5) The purified copolymer was dried under vaccum and characterized by Infrared spectroscopy (IR) and Gel permeation chromatography (GPC).

Infra Red (IR) Spectroscopy & Gel Permeation Chromatography (GPC)
Infrared spectrum of any compound or drug gives information about the groups present in that particular compound. IR spectra (Thermo Nicolet Nexus 670 spectrophotometer). Molecular weight of the copolymers was determined by gel permeation chromatography method (Perkin Elmer Series-200. Tetrahydrofuran (THF) was used as mobile phase and the flow rate was maintained at 1 mL/ min at 30°C.
Preparation of Nanoparticles
Paclitaxel-loaded PEGylated nanoparticles were prepared using emulsion solvent evaporation method which is widely used for the encapsulation of hydrophobic drugs. The organic phase consist of paclitaxel (5 mg) and PEG-PLGA (50 mg) in acetone was added at a constant flow rate (0.3 ml/min) into 20 ml of aqueous phase containing 1% of PVA under intense shear using probe sonicator (Lark Innovative Fine Tecknowledge, Chennai, India). The resultant mixture was further stirred for 2 hours using magnetic stirrer. The organic solvent was then evaporated off under vacuum using a rotavapor (Steroglass, Italy). Finally, the nanoparticles were collected by centrifugation (refrigerated centrifuge, Remi C-24) and washed with water before lyophilization (Heto Dry Winner, Germany). (5)

Paclitaxel loaded plain nanoparticles were similarly prepared for comparison by substituting the copolymer conjugate with PLGA. To investigate the fluorescent studies, fluorescein dye-loaded nanoparticles were prepared by substituting the drug with Rhodamine 6G.

Receptor coupling with PEGylated nanoparticles
Transferrin receptors are expressed on highly proliferating cancer cells and more profusely on endothelial cells of the BBB as compared to endothelial cells at other vicinities in the body. Transferrin receptor appended PEGylated nanoparticles was prepared by incubating oxidized Tf with 1 ml of PEGylated nanoparticles. After 1.5 hours at room temperature, 50 μl of glycine solution was then added and the Tf-PEG-nanoparticles were purified to remove excess free Tf by centrifugation at 39,000 x g for 20 minutes and the protein concentration was determined using bicinchoninic acid (BCA) kit.

III. CHARACTERIZATION OF NANOPARTICLES

Shape morphology
Various nanoparticles (NPs) were characterized for their shape morphology using transmission electron microscopy (TEM) using a Philips CM12 Electron Microscope, (Eindhoven, Netherlands).

Particle size & Zeta Potential
Particle size of the nanoparticles was determined with the help of laser diffraction particle size analyzer (Cilas 1604L, France). Plain, PEGylated and transferrin coupled PEGylated nanoparticles were suspended in the chamber of particle size analyzer containing milli-Q water and the vesicles size was determined using the software provided with the instrument. Zeta potential of the various nanoparticles was determined using Zeta Sizer (Zetasizer 3000; Malvern, UK). The nanoparticles were suspended in 1mM HEPES buffer (Sigma, USA) and adjusted to pH 7.4 by 0.1 M HCl.

Percent drug entrapment (PDE)
Lyophilized plain, PEGylated and transferrin coupled PEGylated nanoparticles were digested in 5 ml of 0.1M NaOH at 50 °C for 10 min to release the drug content and resultant mixture was filtered.(6) The amount of drug was quantified by HPLC method reported in I.P. (2000) for paclitaxel. (7)

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\text{Amount of drug released from the lysed NPs} = \frac{\text{Amount of drug initially taken to Prepare the NPs}}{100}
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In vitro drug release
In vitro drug release from the plain, PEGylated and transferrin coupled PEGylated nanoparticles bearing paclitaxel was studied using dialysis bag. Various nanoparticles formulations free from unentrapped drug (1 ml of 40 mg nanoparticles) were taken into a dialysis bag (MWCO 3500, Himedia, Mumbai) which was suspended in a beaker containing 50 ml of saline phosphate buffer (pH 7.4). The contents of the beaker were shaken using a magnetic stirrer at 37 ± 1°C. 1 ml of the sample was withdrawn periodically and replaced with the same volume of fresh saline phosphate buffer pH 7.4 and the amount of drug was quantified by HPLC method.

In vitro cytotoxicity
In vitro cytotoxicity assay was done on 8 human cancer cell lines of 6 different human cancer tissues viz prostate (DU145, PC3), Colon (COLO-205, HCT 15), Breast (MCF-7), Neuroblastoma (IMR-32), CNS (SK-NS-H) and Lung (A549) at various concentration i.e. 10, 30 and 100 μg/ml by Sulforhodamine B (SRB) assay method was used for in vitro cytotoxicity screening, which is a widely used method by National Cancer Institute (NCI), Fredrich, USA.

Fluorescence microscopy
Fluorescence microscopy was performed to confirm the uptake of fluorescence marker (Rh6G) which is lipophilic in nature was encapsulated in various nanoparticles. Rats were sacrificed after 2 hours and brain microtome was prepared.

These micromtomed sections were optically scanned at excitation and emission wavelength of 480 and 520 nm respectively. These studies were approved by Institutional Animal Ethical Committee of Department of Pharmaceutical Sciences, Dr. Hari Singh Gour University, Sagar (MP) and performed according to the norms assigned by CPCSEA.
IV. RESULTS AND DISCUSSION

Infra Red Interpretation & Gel Permeation chromatography
The PEG-PLGA copolymer was synthesized using carbodiimide chemistry. The IR spectra for the conjugate of PEG-PLGA exhibited that COOH groups of PLGA conjugated with NH₂ group of PEG as amine peaks at 3525 cm⁻¹ on IR spectra. The bands at 3010 and 2950 cm⁻¹ are due to C-H stretch of CH₃ and 2880 cm⁻¹ due to the C-H stretch of CH₂. A stretch at 1762 cm⁻¹ shows C=O stretch (Fig. 1). The average molecular weight was found to be 50800 with a unimodal mass distribution that excluded the presence of PEG and PLGA.

Preparation of Nanoparticles
Paclitaxel loaded plain and PEGylated nanoparticles were prepared by emulsification solvent evaporation method. The technique relies on the rapid diffusion of the solvent from the internal phase into the external phase, which thereby provokes polymer aggregation in the form of solid colloidal particles. Conventional PLGA (Non-PEGylated) nanoparticles were also included in this study for the comparison.

Transferrin estimation
The coupling efficiency was assessed quantitatively by taking average number of transferrin molecules per thousand PEG chains which was calculated by assuming that the molecular weight of Tf is 80,000. It was found that approx 3.45% of the total PEG chains were linked to transferrin molecules.

Shape and surface morphology
Shape and surface morphology of the Tf-PEGylated nanoparticles were evaluated by transmission electron microscopy (TEM). The study reveals that, the nanoparticles are spherical with smooth surface. (Fig. 2).

Particle size & Zeta potential
The average particle size of plain, PEGylated & Tf-PEG- nanoparticles was found to be 138.11±2.4nm, 146.56±1.8nm & 156.45±2.56nm, respectively. The average particle size was increased due to PEGylation for PEGylated nanoparticles and further after PEGylation due to the coupling of transferrin to the surface of PEGylated nanoparticles. Zeta potential measurement was done for plain and PEGylated NPs which confirm the PEGylation on the surface of the nanoparticles. NP prepared from PLGA polymer shows a zeta potential of -37.2±1.3mV, while the PEGylated Nanoparticles shows a low negative zeta potential of -5.1±0.3mV, which could be due to the presence of PEG on surface of the PEGylated NPs.

Percent drug entrapment (PDE)
Percent drug entrapment of plain nanoparticles was found to be 74.2±3.22% and 72.8±2.12% for PEGylated nanoparticles. Percent drug entrapment was decreased from to 69.9±1.56% as on coupling transferrin. This decrease in PDE could be attributed to the residual drug leakage from the NPs during the incubation period employed for coupling of transferrin to the surface of the PEGylated NPs.

In vitro drug release
In-vitro drug release behaviour of Non-PEGylated NPs shows biphasic pattern which is characterized by an initial fast release, followed by a slow sustained release. About 26.41±1.32% TMZ was released within 6 hours and nearly 68.8±2.42% was released at the end of 120 hours. The same pattern of in vitro release was also observed with PEGylated nanoparticles and Transferrin coupled PEGylated nanoparticles. The initial fast drug release i.e., 16.5±2.12% and 7.1±1.58% in 6 hours was observed with PEGylated nanoparticles and transferrin coupled PEGylated nanoparticles, respectively. A sustained drug release was observed reaching a release of 55.6±2.69% for PEGylated nanoparticles and 45.9±2.41% for transferrin coupled PEGylated nanoparticles at 120 hours.

The fast release /burst effect has been attributed to the rapid release of the fraction of drug located on or close to the surface of the NPs. The decrease in the drug release from PEGylated NPs as compared to Non-PEGylated NPs is due to increased drug diffusion barrier of the PEG chains. While the transferrin coupled PEGylated NPs clearly indicate the decrease in release which could be due to the structural integrity conferred by transferrin coupling and might lead to a double barrier effect for drug diffusion. Since the system is based upon ligand mediated localization of the entrapped drug, the suppression of drug efflux should not influence its targeting potential.

In vitro cytotoxicity
Paclitaxel shows more than 68% of the growth inhibition for all cancer cell lines at 80μg/ml, while at 30 & 10μg/ml the inhibition varied between 12-61% and 1-38%, respectively. Plain nanoparticles show lower cytotoxicity than the paclitaxel. While the cytotoxicity result produced by PEGylated and Tf-PEGylated nanoparticles was higher than the plain nanoparticles, which could be due to the slow release of drug for a longer period of time. Nanoparticles escape through endosomes, and the fraction of nanoparticles that escapes the endosomes, retain inside the cell and release the drug slowly, thus sustaining the intracellular drug levels when incubated. Tf-PEGylated nanoparticles show reduced cytotoxicity than uncoupled nanoparticles, which results in greater intracellular retention than uncoupled ones. Since, the receptor transferrin has the ability to recycle within minutes after endocytosis, so by use of single receptor, multiple
intracellular delivery of transferrin coupled PEGylated nanoparticles occurs, thus leading to greater drug delivery.

**Fluorescence Microscopy**

Fluorescence microscopy shows the distribution in blood vessels and accumulation of dye in neurons. The photomicrographs clearly reveal that Tf-PEGylated nanoparticles are crossing the basal carotid system, with a maximum fluorescence intensity in the brain tissues. Further these section (tissue) was scanned in three directions, along the x-, y-, and z-axis. Photomicrograph shows the localization of Tf-PEGylated nanoparticles into the brain tissue. The nanoparticles are adhering to the endothelium of the brain vessels are clearly visible, as well as the marker was diffusing into the brain tissue.

V. CONCLUSION

Receptor liganded long circulating nanoparticles served as a very good potential drug delivery system for transport across BBB through receptor mediated endocytosis. The results revealed that proposed system could be exploited as potential carrier for delivery of drugs to the brain.

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