P-113 **Transferrin appended Long Circulating Nanoparticles for Brain Delivery of Temozolomide**

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INTRODUCTION AND OBJECTIVES

Polymer based nanotechnologies are proposed to be an alternative for drug administration, delivery and targeting to those of conventional formulations. Targeting drugs to the brain is a challenging job because of the presence of the blood brain barrier, which is a rate-limiting factor in determining permeation of a drug into brain. The BBB is very specialized system of endothelial cells that separates the blood from the underlying brain cells, providing protection to brain cells and preserving brain homeostasis. The CNS (or brain) has transport routes that overcome the BBB by other than passive diffusion, such as carrier/receptor mediated transcytosis. These receptors can be used to deliver drugs to the brain. Conventional nanoparticles were rapidly removed after intravenous administration from the blood stream by the macrophages of the mononuclear phagocyte system. This limits their application in the field of controlled drug delivery and drug targeting to the tissues. The presence of the hydrophilic coating on the surface of the nanoparticles is thought to sterically stabilize them against opsonization and phagocytes between the hydrophilic polymers. PEG has been found to be a particularly effective steric stabilizer, probably due to its high hydrophilicity, chain formation, electrical neutrality and absence of functional group, which prevents interactions with biological components *in vivo*. The stability of the PEG surface layer to desorption/displacement *in vivo* is essential for the long circulation effect.

MATERIALS AND METHODS

Temozolomide 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, Polyvinyl alcohol (PVA), human transferrin, 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.

PEG-PLGA Synthesis Copolymer of PEG-PLGA was synthesized using solution polymerization process under nitrogen, where stannous octoate was used as catalyst. Briefly, lactide and glycolide in a molar ratio of 4:1 and the specified amount of Bis-PEG were put in thick-walled glass tubes. The total weight of the feed was about 3gm. Stannous octoate was dissolved in hexane and added at a concentration of 0.03% by the weight of the feed. Then, the tubes were heated at 190°C for the 2 hours. The resulting copolymer was purified by dissolving in chloroform and then precipitating in an excess methanol. The purified copolymer was dried under vaccum and characterized by IR and gel permeation chromatography.

Preparation of Non-PEGylated Nanoparticles Drug loaded Non-PEGylated nanoparticles were prepared using emulsion solvent evaporation method which is widely used for the encapsulation of hydrophobic drugs [9]. Briefly, temozolomide (5mg) and PLGA (50mg) were dissolved in acetone (5ml) (organic phase). The organic phase 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 with continues stirring for 2 hours using magnetic stirrer. The organic solvent was then evaporated off under vacuum using a rotavapor (Steroglass, Italy).

Preparation of PEGylated Nanoparticles PEGylated nanoparticles were also prepared by emulsification solvent evaporation method, all the parameters were kept same except the PLGA polymer was replaced with PEG PLGA Copolymer. Similarly fluorescein dye loaded nanoparticles were also prepared by incorporating Rhodamine 6G (Rh6G) during the preparation of nanoparticles.

Characterization Particle size was determined by laser diffraction particle size analyzer (CILAS, 1064L, France) while shape & surface morphology was examined by SEM. The surface ξ (zeta) potential of nanoparticles was estimated by Zeta Sizer (Zetasizer 3000; Malvern, UK) for assessing the extent of PEG coating on the surface of PEGylated nanoparticles and to investigate whether the drug was encapsulated or simply adsorbed onto the surface. The drug entrapment efficiency was determined by disrupting the nanoparticles, further the *in vitro* drug release was determined using dialysis technique. *In vitro* cytotoxicity studies were performed 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). The qualitatively uptake of the formulations by the brain in albino rats was also assessed by fluorescent & confocal microscopy. Biodistribution study was performed using gamma scintillation technique by measuring the radioactivity in different organs after i.v. administration of the ^{99m}TcTmz bearing formulations. Process parameters were optimized.

RESULTS AND DISCUSSION

The particle size was found to be 110.22±1.2nm and 116.54±2.1nm for Non-PEGylated and PEGylated nanoparticles bearing temozolomide, respectively. Further the size was increased to 120.32±2.42 nm in case of transferrin coupled PEGylated NPs. This could be due to the coupling of –COOH group of transferrin with the NH_2 group present at the surface of the PEGylated NPs which resulted in increased in size.

PEGylated Nanoparticles have a low negative zeta potential value -4.2±0.1mv as compared to Non-PEGylated NPs (-35.6±0.3mv) because the carboxylic acid end groups of PLGA are capped by the PEG segments. The protective (stealth) action of PEG is mainly due to the formation of a dense, hydrophilic cloud of flexible chain on the surface of the particle that reduces the hydrophobic interaction with the RES and enhances the circulation time.

The PDE was found to be 77.21±2.11% for Non-PEGylated nanoparticles and 75.89±1.88% for PEGylated nanoparticles bearing temozolomide. While the PDE was decreased to 70.44±2.12% as on coupling transferrin at the surface of PEGylated nanoparticles which is attributed to the residual drug leakage during the incubation period employed for coupling of transferrin to the surface of the PEGylated NPs.

Non-PEGylated, PEGylated and transferrin coupled PEGylated NPs were subjected to *in vitro* drug release studies in saline phosphate buffer pH 7.4 which shows biphasic pattern of an initial fast release, followed by a slow sustained release. 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 double barrier effect for drug diffusion.

Temozolomide showed more than 70% growth inhibition against all the cancer cell lines at 100µg/ml, while 10-60% and 0-48%, inhibition at lower concentration of 30 and 10µg/ml, respectively. Non-PEGylated nanoparticles have shown lower cytotoxicity then the plain drug, while cytotoxicity produced by PEGylated and Tf coupled PEGylated nanoparticles was slightly lower then the Non-PEGylated NPs. This could be attributed due to slower release of drug from the PEGylated and Transferrin coupled PEGylated nanoparticles in the culture. Although, nanoparticles escape through endosomes, a major fraction of it probably remains in the recycling endosomes and undergoes rapid exocytosis.

The confocal laser scanning microscopy studies of brain tissue after the administration of nanoparticulate formulations showed enthusiastic results. Microscopic observation of coronal sections of the mouse brain showed that green particles of the transferrin coupled PEGylated nanoparticles distributed extensively in the periventricular region of the third ventricle, cortex and striatum while the fluorescence of Non-PEGylated in these regions was almost undetectable.

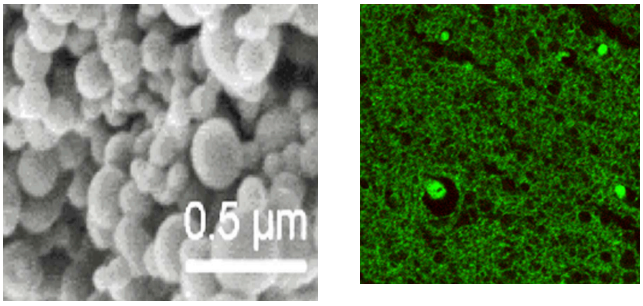

Figure 1: SEM & CLSM of Tf coupled PEGylated Nanoparticles

Biodistribution study results clearly reveal that Plain NP was taken by the RES therefore a maximum accumulation of ^{99m}Tc in liver was observed. While PEG-PLGA-NP there was a decreased uptake by RES which could be due to the coating of hydrophilic coating of PEG on the surface of the nanoparticles. There was increased radioactive accumulation in brain of Tf-PEG-PLGA-NP, which was nearly 7 times higher than the plain NP and about 3 times higher than the PEGylated nanoparticles. This can be attributed to the abundance of the transferrin receptor on the broad variety of tissue in less concentration compared to the brain.

CONCLUSIONS

In conclusion, our developed drug delivery system, for first time proved efficacy of DOX in micro particulate delivery system against treatment of VL using targeted drug delivery approach. Secondly, by exploiting passive and active targeting modes, total dose required for therapy was grossly cut down to few orders less, as evidenced from low dose administration (in µg), rendering the therapy favorable and affordable to common people.
In conclusion, Transferrin appended PEGylated nanoparticles served as a potential system for transporting the drug molecules across the BBB through receptor mediated endocytosis. The present results revealed that receptor coupled long circulating nanoparticles could be exploited as potential carrier for delivery of drugs to the brain.

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