DEVELOPMENT, CHARACTERIZATION AND *IN VITRO* EVALUATION OF LACTOFERRIN CONJUGATED AND MEMANTINE LOADED PEG-PLGA NANOPARTICLES FOR THE TREATMENT OF ALZHEIMER'S DISEASE

ABSTRACT

Alzheimer's disease is a degenerative neurological ailment with no cure and only a limited number of therapeutic options. It has a detrimental effect on cognitive and behavioral capacities. Conventional therapies, such as acetylcholinesterase inhibitors, typically fail to work because they cannot cross the blood-brain barrier. To increase the efficacy of Alzheimer's disease treatment, targeted treatment techniques utilizing nanoparticulate drug delivery devices have been used. Memantine is a medication that has been licensed for the treatment of mild to moderate Alzheimer's disease. We used a double emulsion approach to create lactoferrin (Lf) coupled with biodegradable PEG-PLGA nanoparticles (NPs) to increase memantine's effect at the target site. The synthesized NPs had an average particle size of 162.6±0.5 nm, a polydispersity index of <0.1, and a surface charge of -21.5 mV. The physicochemical characterization of NPs established that the crystalline drug was dispersed within the PLGA matrix. The developed nanoparticulate formulation demonstrated a sustained release profile of memantine during in vitro dissolution experiments. The NPs were noncytotoxic to brain cell lines, and a higher quantity of Lf-NP was found in bEnd.3 cells than with unconjugated nanoparticles. The study established that Lf-conjugated PEG-PLGA nanoparticles containing memantine are suitable for targeted delivery in Alzheimer's disease.

Keywords: Alzheimer's disease, bEnd.3, lactoferrin, memantine, PLGA nanoparticles, PEG.

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia among senior citizens [1]. This disease, characterized by a progressive impairment of cognitive and non-cognitive processes, is devastating for patients, families, and society. Numerous neurotransmitters are involved in this chronic and progressive neurological condition, and the relative contribution of each neurotransmitter to clinical findings is not well understood. Because of the blood-brain barrier, AD and other neurodegenerative diseases are difficult to treat (BBB) [2]. Specific permeability of the BBB is the most significant hurdle to treating AD [1]. Due to the BBB's restriction on drug

distribution across the brain, currently, there are only a few drugs available to treat AD. While the BBB prevents the entry of neurotoxic xenobiotics into the CNS, it also significantly inhibits the entry of neuroprotective medicines into the CNS. To overcome the BBB, either the drug's physicochemical properties must be altered to make it lipid-soluble, or its size must be lowered to a small scale [3-4].

The current therapy strategy for Alzheimer's disease (AD) is based on vascular prevention and symptomatic treatment with cholinesterase inhibitors and N-methyl-D-aspartate (NMDA) antagonists. Memantine is a non-competitive glutamate receptor antagonist [5]. Excessive activation of neuronal amino acid receptors causes glutamate-related excitotoxicity, which contributes to the pathogenesis of Alzheimer's disease [6]. Memantine exerts its effect on the glutamatergic system by blocking NMDA receptors, hence decreasing glutamate activity in brain cells and suppressing neurotransmitter function. Memantine's interaction with NMDA receptors is important for the drug's therapeutic efficacy in AD. However, consuming memantine may cause dizziness, disorientation, constipation, and vomiting [7].

Engineered nanoparticles (NPs) with novel physicochemical properties and the ability to cross the BBB may be a viable method for overcoming biological and pharmacological challenges associated with treating Alzheimer's disease [8]. The key advantage of nanoparticles in treating Alzheimer's disease is the targeted delivery of drugs [9]. PEGylation of the NPs enhances their retention further by making them long-circulating [10]. In addition to this, ligand conjugation is a highly effective method for increasing the targeting efficiency of NPs [11]. Lactoferrin (Lf) is a promising targeting molecule with the potential to enhance brain delivery. Lf receptors (LfR) are located on the BBB and are responsible for the transport of Lf across the BBB [12]. It is also reported that Lf has a substantially higher brain uptake than transferrin and OX26 [13]. Hence the objective of the present study was development and characterization of Lf conjugated PEG-PLGA nanoparticles loaded with memantine for the treatment of Alzheimer's disease.

Methods

Materials

PEG-PLGA polymer and Lactoferrin was obtained from Sigma-Aldrich and memantine (MEM) was procured from Dellwich Healthcare LLP, Ahmedabad. All tests were conducted with water filtered through the Millipore MilliQ system, and all other reagents were of analytical quality.

Preparation of memantine loaded PEG-PLGA nanoparticles

50mg of PLGA–PEG was dissolved in ethyl acetate (5ml) forming the organic phase [14]. Aqueous phase was obtained by dissolving MEM in deionized water. The aqueous phase was added into the organic phase at a constant flow rate under intense shear using probe sonicator to form the primary emulsion [15]. The resultant mixture was then dispersed in 2 ml of deionized water containing PVA (0.3%) and stirred for 2 hours using magnetic stirrer to stabilize the colloidal system [16-17]. The organic solvent was then evaporated off under vacuum using a rotavapor (Steroglass, Italy) and NPs were washed by centrifugation at 15,000 r.p.m. for 20 min. The loading of NPs with rhodamine followed the same procedure [18].

Preparation of memantine loaded Lf-PEG-PLGA nanoparticles

To prepare the Lf-PEG-PLGA NPs, purified thiolated Lf was added to the PEG-PLGA NPs and incubated at room temperature for 9 hours. After passing the solution through a 1.5 cm x 20 cm sepharose CL-4B column, it was eluted with 0.01 M phosphate buffered saline (PBS) buffer pH 7.4 to remove the unconjugated thiolated Lf [19].

Particle size, morphology and Zeta potential

The size of the nanoparticles was determined with the help of laser diffraction particle size analyzer (Cilas 1604L, France). Prepared nanoparticles were suspended in the chamber of particle size analyzer containing milli-Q water and the vesicles size was determined [20]. A ZetaSizer Nano ZS (Malvern Instruments) was used to measure NP zeta potential and polydispersity index (PI) using photon correlation spectroscopy (PCS) [17]. A transmission electron microscope (TEM, Morgani 268D, Holland) was used for the morphological examination of the nanoparticles after staining with 1% (w/v) phosphotungstic acid solution [21-22].

Encapsulation efficiency:

The amount of drug encapsulated in nanoparticles was determined indirectly. Previously to the analysis, the non-loaded drug was separated from NPs by centrifugation at 14,000 rpm and filtered through 500Da MWCO. The encapsulation efficiency (EE) was calculated by the difference between the total amount of drug and the free drug, present in the filtered fraction [23].

In vitro drug release

In vitro drug release of MEM from PEG-PLGA NPs and Lf conjugated PEG-PLGA Nps was studied against free MEM in phosphate-buffered saline (PBS) [17]. Briefly, a volume of 5 ml of

each formulation was placed directly into a dialysis bag (cellulose membrane, 500 Da Himedia, Mumbai) and each bag was placed on 100 ml of PBS pH 7.4 at 37 °C. 1 ml of sample was removed from the stirred release medium at predefined intervals and replaced with 1 ml of new buffer at the same temperature. HPLC was used to determine the amount of drug released at each time point [22].

In vitro cellular uptake of drug-loaded PEG-PLGA NPs and Lf conjugated PEG-PLGA NPs

Cell culture

Dulbecco's Modified Eagle Medium containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml) was used to culture the immortalized mouse brain endothelial cell line b.End3 in 10 cm tissue culture dishes.

Cellular uptake and competition assay of MEM loaded Lf conjugated PEG-PLGA NPs and PEG-PLGA NPs

bEnd.3 cells were seeded at a density of 105 cells/cm2 onto 24-well plates. A suspension of nanoparticles (1–60g/ml) was added to the pre-incubated cells with HBSS for 15 minutes on the second day and incubated for 1 hour at 37oC. The analysis for done for uptake of nanopartuculate formulations. For the competition assay, bEnd.3 cells were treated with Lf in RPMI solution (1 mM) and incubated for 30 min in advance. Afterwards, Lf treated and untreated cells were exposed to rhodamine loaded Lf conjugated PEG-PLGA NPs and maintained for 2 h at 37oC. The cells only with Lf solution were utilized as a blank control. Then the cells were washed three times with PBS (pH 7.4) subjected to observe with a Confocal Imaging microscope (FV3000, Olympus) [23].

RESULTS AND DISCUSSION

The double emulsion evaporation process was adopted for the fabrication of PLGA NPs because it is well suited for loading hydrophilic drugs such as MEM. The active Lf on the surface of the nanoparticles would ensure that the nanoparticles were targeted to the Lf receptor on brain capillaries. Knowing that the mean particle size is a key parameter for NPs to pass through the BBB, the purpose of this study was to produce NPs with a mean particle size of 100 and 200 nm. The average particle size of drug loaded PEG-PLGA NPs was found to be around 120 nm with the zeta potentials of around −21.5 mV. After Lf conjugation the nanoparticle size raise to around 162.6±0.5 nm (Figure 1). The polydispersity index for formulation was found to be < 0.1

showing a monomodal distribution. The size of the prepared NPs was all below 200 nm that was regarded as favorable to brain transport.

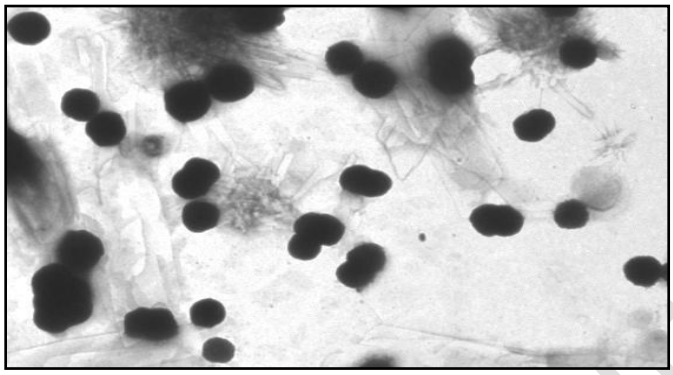

Figure 1: TEM photograph of MEM loaded Lf conjugated PEG-PLGA NPs

TEM images revealed that the Lf conjugate PEG-PLGA NPs had a spherical shape. The results of in vitro release investigation done at a temperature of 37oC in PBS pH 7.4. Following the initial burst phase, the drug released slowly from the polymeric matrix into the release medium. The initial burst release of drug could be attributed to the unloaded MEM portion, which is only weakly bound to the surface of the NPs due to the PEG coating. In vitro drug release study also shown that around 66.82% of MEM was releases in 24 hrs from MEM-loaded PEG-PLGA NPs and 58.95% of MEM was releases in 24 hrs from Lf conjugated PEG-PLGA NPs, confirming the slower release of the drug from the prepared nanoformulation (Figure 2).

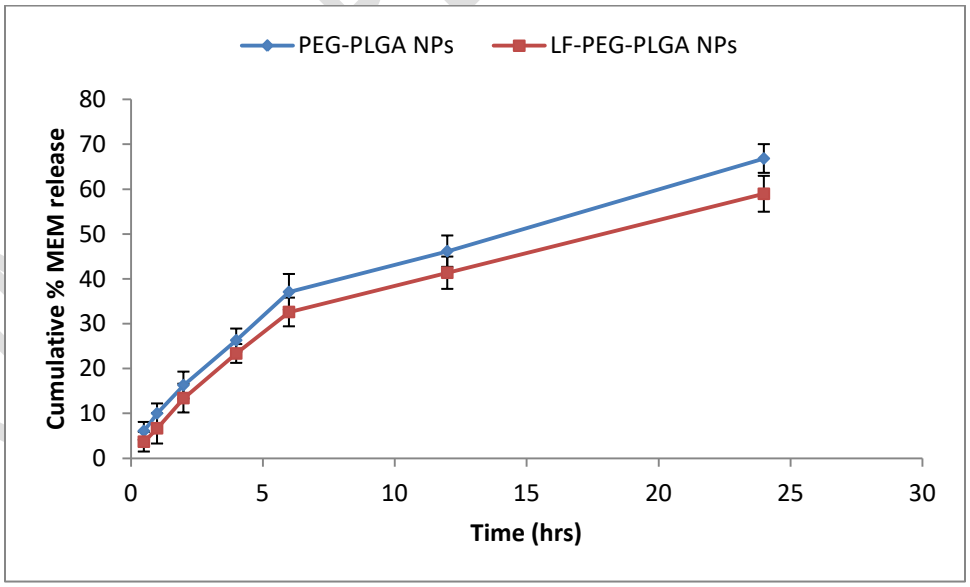

Figure 2: In vitro drug release from prepared nanoparticles. ±SD (n=3)

The cellular uptake of prepared nanoparticles in bEnd.3 cells were investigated to evaluate the

targeting potential of the formulation. As a model for the BBB, bEnd.3 cells are a good choice because of their rapid growth, capacity to maintain blood-brain barrier properties through repeated transit, creation of functional barriers, and openness to a wide range of molecular treatments. A concentration-dependent in vitro uptake result for rhodamine-loaded Lf-NP by bEnd.3 cells indicated an endocytosis process. The uptake of Lf conjugate PEG-PLGA NPs by bEnd.3 cells was higher than the uptake of PEG-PLGA NPs. The uptake of Lf conjugate PEG-PLGA NPs increased with increase in the concentration.

In the competition assay, the cellular uptake of Lf conjugate PEG-PLGA NPs formulation was significantly higher than PEG-PLGA NPs formulation. After presaturation with free Lf, the fluorescence intensity of cells incubated with Lf conjugate PEG-PLGA NPs formulation was reduced, indicating that the decreased cellular uptake of Lf conjugate PEG-PLGA NPs formulation was due to free Lf binding competitively to receptors on bEnd.3 cells, further confirming Lf targeting effect on bEnd.3 cells via receptor mediated endocytosis.

CONCLUSION

Developing drug carriers with a wide range of features is now possible because of advancements in nanotechnology. These nanosystems could be used to deliver medicines and other neuroprotective drugs to the brain more effectively in treating Alzheimer's disease. In this study, a novel surface engineered brain drug delivery system was developed with an average size lower than 200 nm and PI < 0.1, characteristic of monodispersed systems, suitable to release the drug across the BBB. Developed formulation shown a sustained release profile of memantine. The significantly increased uptake of the Lf conjugate PEG-PLGA NPs by bEnd.3 cells compared with that of plain PEG-PLGA NPs was confirming the brain targeting potential of the developed carrier. In summary, MEM loaded Lf conjugate PEG-PLGA NPs could be a promising alternative towards a better treatment of AD patients since NPs have demonstrated to be capable to provide a more effective treatment than free MEM.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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