**Executive Summary of the Ph.D. Thesis entitled**

# "Development of Nanocarrier based Targeted Drug Delivery System for Effective Treatment of Brain Tumor"

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## 1.0 INTRODUCTION

### 1.1 Brain tumor

Brain tumors refer to a heterogeneous group of primary and metastatic neoplasms in the central nervous system and are one of the life-threatening diseases which are characterized by low survival rate (1). According to GLOBOCAN 2018, nearly 296,851 new cases of brain and nervous system tumors and 241037 deaths are diagnosed in 2018 worldwide. In India 28142 new brain tumor cases annually are reported while deaths were 24003 in 2018 (2). The estimated incidence, mortality and 5 year prevalence of brain tumor among men in India is approximately 11855, 9574 and 17251 respectively which represents 2.5%, 2.7% and 2.6% of Indian population respectively while for women it is 6976 (1.3%), 5578 (1.7%) and 10157 (1%) respectively. More than 120 types of brain tumors are identified till date and depending on the origin of tumor, most common tumors are grouped as tumors of neuroepithelial tissue, tumors of cranial and spinal nerve, tumors of meninges, heamopoitic origin neoplasm and lymphomas, tumor of sellar region, germ cell tumors and cysts (3,4). The most prevalent brain tumors are intracranial metastases from systemic cancers, meningiomas, and gliomas, specifically, glioblastoma (1,5). Glioma is the most frequent primary brain cancer which accounts for 29% of all primary brain and CNS tumors and 80% of malignant brain tumors. These malignant gliomas are primary tumors that are derived from glial origin and account for approximately 70% of new primary brain cancer diagnosis. The classification, grading, and treatment of this diverse group of tumors have been primarily based on morphological criteria, which introduced a certain degree of interpretative subjectivity and moreover provided only suboptimal accuracy for the prediction of treatment response (6). WHO has classified glioma in three category viz. astrocytoma, oligodendrogliomas and mixed gliomas (oligoastrocyotomas). Amongst gliomas, glioblastomamultiforme (GBM) which is a grade IV astrocytoma according to the World Health Organization (WHO) classification, is the most common and aggressive form of glioma in nature (3,6). The median survival for glioblastoma is 14 months (7). The high mortality rate due to GBM can be attributed to specific properties of glioma which includes highly infiltrative nature and lack of clear margin. The existing therapy for GBM is nonspecific and almost fails to prevent reoccurrence of disease.

## 1.2 Current treatment approaches for brain tumor

Multiple treatment approaches have been experimented for treatment of brain cancer and may vary depending on the type, grade, size and location of the tumor; whether it has spread; and patient age and general health. The multidisciplinary approach for treatment of brain tumor includes combination of surgery, radiation and chemotherapy (3).

Most common initial therapy for brain tumor is chemotherapy. Various therapeutic moieties indicated in brain tumor are available in oral and parenteral dosage form in market. Temozolomide is the first generation drug used for the treatment of brain tumors and is presently given orally (8). Other therapeutic drugs indicated for being used in treatment of brain tumor include irinotecan, carmustine, cisplatin and lomustine. Most of the drugs have shown enhanced anticancer activity for brain tumor *in vitro*. However, clinical failure was observed with such drugs due to insufficient barrier passage.

Although chemotherapy in combination with other treatment approaches such as radiotherapy and surgery has proved effective still tumor recurrence (96% cases) adjacent to resection margin after surgical resection makes it important to develop adjuvant therapy which can help minimize the recurrence with enhanced efficacy and specificity. Poor prognosis and rapid recurrence are associated with standard therapy of glioma because infiltrate growth of gliomas makes it difficult for the surgeon to completely remove pathologic or cancer-infiltrated tissues without affecting normal brain functions. Furthermore, the failure is also ascribed to the side effects of radiotherapy and poor outcome of usual chemotherapy (9).

## 1.3 Limitations associated with chemotherapeutic agents

The major hindrance to prognosis of brain tumor is the auto protective nature of the brain (BBB and alignment of brain cells), genomic alterations occurring in tumor cells, efflux transporters on the barrier and properties of chemical agents used for treatment of brain tumors. Brain allows passage of some of endogenous material, a few hydrophobic agents and particles with molecular weight of less than 500Da. Lipophilicity of the drug is one of the important factors that should be considered while designing new entities for treatment of tumors. Improvement in passage of drug across the BBB is possible with increased lipophilicity but this may be associated with increased drug uptake by other tissues, causing an increased tissue burden. This non selectivity in delivery of drugs to non targeted site is detrimental; especially

when cytotoxic agents are used, because drug toxicity would be higher at non target sites. Enhanced efflux along with loss of CNS activity is another major drawback of increased lipophilicity which might lead to poor tissue retention and short biological action. Improvement in the therapy could be brought about by modulating the pharmacological properties of the drug (10).

### 1.4 Nanoparticles as drug delivery platform for overcoming drawbacks of conventional therapy

In past several decades, nanoparticles have gained lots of attention for treatment of cancer and were fabricated for cancer treatment by modulating their physicochemical properties including composition, size, shape and surface modification. Nanoparticles have potential to overcome the drawbacks of conventional cancer chemotherapy because of unique properties like small size, surface charge, variable shape, several binding sites for the attachment of target specific ligands, antibodies, peptides etc. They can also enhance the tumor targeting by both passive and active targeting mechanism. Passive targeting is possible due to enhanced permeability and retention (EPR) effect (11,12). Nanoparticles based delivery systems are also approved by the FDA for clinical use (Abraxane, Doxil, Genexol-PM, DepoCyt, Myocet etc.) and many more are in the clinical trials (NK105, CYt-6091, Genexol-PM, Rexin-G etc.) (11,13). As compared to conventional chemotherapy, nanoparticles based delivery systems have several advantages and features, including: 1) improved delivery of poorly water soluble drugs, peptides, and genes; 2) better protection of drugs, peptides or genes from harsh environments (e.g., enzymatic degradation and the highly acidic environment in the lysosomes or stomach); 3) enhanced treatment efficiency and reduced systemic side effects by cell- or tissue specific targeted delivery of drugs, peptides or genes; 4) overcome multidrug resistance by co delivery of drugs, peptides, genes and/or diagnostic agents; 5) stimuli-responsive systems (pH sensitive, temperature sensitive, redox sensitive) can control release of drugs, peptides or gene over a manageable period of time at precise doses (11,14).

Nanoparticles used as a carrier for cancer therapeutics may be of several types viz. protein based nanoparticles (albumin nanoparticles, gelatin nanoparticles etc.) (15,16), polymer based nanoparticles (poly lactide co glycolide nanoparticles, polycaprolactone nanoparticles, polylactide nanoparticles, chitosan nanoparticles etc.) (17–20), lipid based nanoparticles (solid

lipid nanoparticles, nanostructured lipid carriers, liposomes etc.) (21–23), lipid polymer hybrid nanoparticles (24), metal nanoparticles (25–27), polymeric micelles (28), dendrimers (29) etc. Among all these nanoparticles, protein based nanoparticles have gained much more attention in cancer therapy due to unique properties viz. relatively safe and easy to prepare, capability to deliver proteins, peptides, genes, nucleic acid, and hydrophilic as well as hydrophobic anticancer molecules, site specific targeting by surface modification, greater stability profile during storage, etc. (30).

### 1.5 Albumin nanoparticles

Albumin based nanoparticles are utilized for cancer treatment as they are biodegradable, non antigenic and can be also surface modified which may help in avoiding the undesirable toxicity of drugs by modifying their body distribution and improve their cellular uptake. They also have targeting potential because proteins themselves act as passive as well as active targeting moiety. Other targeting ligands can also attach in these carriers to provide site specificity (31). Albumin nanoparticles can be prepared by several methods like desolvation, emulsification, thermal gelation, nano spray drying, nab technology and self assembly etc. The selection of the method is based on several factors such as type of system, area of application, required size, type of drug (hydrophilic or hydrophobic), etc (31). Role of albumin nanoparticles and their applications in cancer therapy will be discussed in the subsequent chapter of the thesis.

### 1.6 Surface modification of albumin nanoparticles for targeted delivery to brain tumor

Surface modification of albumin nanoparticles is necessary to alter the surface properties and enhance the targeting potential of the delivery system. Presence of different binding sites and functional groups like carboxyl and amino groups on albumin offers several possibilities for surface modification of albumin nanoparticles. Surface modification of albumin nanoparticles with the specific ligand can be done by conjugating functional group of albumin with the ligand by covalent bond. For surface modification of albumin nanoparticles, electrostatic adsorption or surface coating techniques may be utilized as non covalent attachment of ligands. In surface modified albumin nanoparticles, albumin plays a role of carrier for delivering therapeutic moiety whereas the ligand is used to modify the pharmacokinetic parameters, improve stability,

prolonging circulation half life, modifying the release pattern of therapeutic moiety or as a targeting agent (16,32).

### 1.6.1 Hyaluronic acid as targeting ligand

HA is natural, anionic, non-sulfated glycosaminoglycan that consists of β-1,4 linked D-glucuronyl-β-(1,3) (Gln)-N-acetyl-D-glucosamine and are widely distributed throughout epithelial, neural and connective tissues (33). HA is the largest polysaccharide in the body, with an average molecular weight of 1-8 MDa (34,35). Human skin also contains large amount of HA i.e. 400-500 µg HA/g (36). In other organs, the content of HA can vary from approximately from 1 to 100 µg HA/g (37). HA plays significant role in various biological processes, cancer metastasis, cell migration, cell differentiation and wound healing (38). Additionally, CD44, a glycoprotein, is HA receptor and are over expressed in large number of mammalian cells and its interaction with HA is crucial for the growth and metastasis of cancer cells (39). A lot of attention has been attracted by the researchers towards investigation of HA as a targeting moiety in cancer therapy and cancer imaging which will be discussed in subsequent chapter of the thesis.

### 1.6.2 Chondroitin sulfate as targeting ligand

Chondroitin sulfate (CS), type of glycosaminoglycans, consists of disaccharide units of b-1,3-linked N-acetyl galactosamine and b-1,4-linked d-glucuronic acid with certain sulphated positions (40). CS is widely distributed in mammals' skin, cartilage, bones and blood vessels (41–44). Being similar to HA, CS has also been reported to have the ability to recognize and interact with HA-mediated CD44 receptors.

### 1.6.3 Lactoferrin as targeting ligand

Lactoferrin (Lf) is an 80 kDa cationic protein belonging to transferrin family which shows 60-80% of sequence similarity with transferrin (45). In another way, Lf is mammalian, cationic iron-binding glycoprotein which consist of polypeptide chains of about 690 amino acids folded into two globular lobes, each of which consist of one iron-binding site (46). The various studies demonstrated that therapeutic moiety loaded nanoparticles when conjugated with Lf were efficiently crosses BBB as compared to transferrin as targeting moiety as well as non-conjugated nanoparticles. Additionally, Lf receptors not only present on BBB but also present or over

expressed in glioblastoma cells (47,48). The finding revealed that Lf can be a good candidate for crossing BBB and ultimately targeting brain tumor.

## 1.7 Aims and objectives

The aim of the present research work was development of nanocarrier based targeted drug delivery system for effective treatment of brain tumor. The proposed study was planned to achieve an effective and selective brain tumor targeting using albumin nanoparticles as drug delivery platform with two model drugs (temozolomide and lenalidomide). It would help to inhibit the growth of tumor by targeting therapeutic moiety to tumor so as to prevent metastasis and growth of tumor. Further for making albumin nanoparticles target specific, surface modification was done with suitable targeting ligands (HA, CS and LF) for selective brain tumor targeting that would help to reduce the toxicity associated with anticancer therapeutic moieties.

Thus the present research work was proposed to be carried out in following steps.

**Step 1:** Selection of suitable method for preparation of nanoparticles (albumin) based on the properties of drugs.

**Step 2:** Optimization of prepared nanoparticles based on experimental design.

**Step 3:** Surface modification of nanoparticles with suitable targeting ligands and their optimization.

**Step 4:** Characterization and evaluation of developed nanoparticles.

**Step 5:** In vivo studies

**Step 6:** Stability studies

## 1.8 Hypothesis

It was hypothesized that incorporation of selected drugs into albumin nanoparticles will prevent drug from external physiological environment and surface modification with specific ligand will help in brain specific drug delivery by receptor mediated targeted drug delivery. The surface modified nanoparticles will lead to enhanced delivery of the drug to the brain by prevention of the clearance by reticuloendothelial system and probable inhibition of the efflux mechanism of

brain and ultimately enhance drug concentration towards target site. This targeted drug delivery will lead to reduction in dose and also will reduce drug related toxicity towards normal cells.

### 1.9 Plan of work

1. Procurement of chemicals and therapeutic moiety.
2. Fabrication of albumin nanoparticles.
3. Optimization of albumin nanoparticles.
4. Drug loading and optimization.
5. Characterization of developed albumin nanoparticles viz. particle size, PDI, zeta potential, percent entrapment efficiency, percent drug loading efficiency, Infrared spectroscopy, Differential scanning Calorimetry (DSC), X-ray diffraction study (XRD) etc.
6. Surface modification of developed drug loaded albumin nanoparticles using different targeting ligands.
7. Optimization of surface modification on the basis of different quality attributes like particle size, PDI, zeta potential and percent conjugation efficiency.
8. Characterization of surface modified albumin nanoparticles viz. particle size, PDI, zeta potential, percent entrapment efficiency, percent drug loading efficiency, percent conjugation efficiency, Infrared spectroscopy, Differential scanning Calorimetry (DSC), X-ray diffraction study (XRD) etc.
9. *In vitro* drug release studies.
10. *In vitro* cell line studies.
11. *In vivo* studies.
12. Stability studies

## 2.0 Research methodology

### 2.1 Literature review

Extensive literature review was carried out through peer reviewed journals available on the topic, books, patents and internet database.

### 2.2 Selection of drug

Two different anti cancer agents, temozolomide (TMZ) and lenalidomide (LND), were selected based upon the literature review, physico-chemical properties and suitability of the molecule to be entrapped in the nanocarrier.

### 2.3 Analytical methods

Various analytical methods like UV-visible spectrophotometry, High pressure liquid chromatography (HPLC) and spectrofluorometric methods were used to estimate the drug and other excipients. For estimation of TMZ inside the nanocarrier and for other *in vitro* studies UV-visible spectrophotometry method was utilized while estimation of TMZ in plasma and organ homogenates were carried out using HPLC. In case of LND, UV-visible spectrophotometry, and spectrofluorometric methods were utilized for estimation of LND inside nanocarrier and for other *in vitro* characterizations. Apart from drugs, for the estimation of other excipients like HA, CS and Lf, CTAB turbidimetric method (HA and CS) and Bradford assay (Lf) were utilized.

### 2.4 Drug – Excipient Compatibility Studies

Compatibility studies of selected drug candidate and excipients were investigated using DSC and FTIR. Pure drug, excipients and physical mixture of the drug and excipients were subjected to such study to determine the compatibility between the drug and selected excipients.

### 2.5 Formulation development and optimization

Drug (TMZ and LND) loaded albumin nanoparticles (TNPs and LNPs) were prepared using desolvation method. The optimization of TNPs was carried out using OVAT analysis followed

by Box-Behnken response surface methodology while for optimization of LNPs OVAT analysis method was utilized.

### 2.6 Surface modification of developed TNPs and LNPs with different targeting moieties

For surface modification of TNPs, two different targeting moieties HA and CS were used to target the over expressed CD44 receptors while for surface medication of LNPs, Lf was used to target over expressed lactoferrin receptors. The conjugation of HA/CS with TNPs were carried out using carbodiimide chemistry to get HA-TNPs and CS-TNPs. The percent conjugation efficiency of HA and CS was determined using CTAB turbidimetric method. For getting Lf-LNPs, Lf was physically adsorbed over LNPs utilizing electrostatic interaction between Lf and LNPs and percent modification efficiency was determined using Bradford assay.

### 2.7 Characterization of developed albumin nanoparticles

The developed albumin nanoparticles (TNPs, HA-TNPs, CS-TNPs, LNPs and LF-LNPs) were characterized using different techniques like particle size, PDI and zeta potential analysis, DSC, FTIR, XRD and TEM analysis.

#### 2.7.1 Particle size and PDI

The particle size and PDI of developed albumin nanoparticles were determined by photon correlation spectroscopy (PCS) with a Malvern Zetasizer (Nano ZS, Malvern ltd., UK). Prior to the measurements, all samples were diluted with double distilled water to produce a suitable scattering intensity. The z-average and PDI values were obtained using disposable polystyrene cells having 10 mm diameter cells at 25°C, which were equilibrating for 120 seconds. Refractive index (RI), for size measurement of NPs dispersion, was set as RI = 1.330 (abs = 0.01). All measurements were performed in triplicate at 25°C (49).

#### 2.7.2 Zeta potential

The zeta potential, reflecting the electric charge on the particle surface and indicating the physical stability of colloidal systems, of developed albumin nanoparticles were measured by determining the electrophoretic mobility using the Malvern Zetasizer (Nano ZS, Malvern ltd., UK). The measurements were performed after diluting in samples with double-distilled water.

Zeta potential was measured using Dip cell with applying field strength 20 V/cm and the average of the zeta potential was given from 30 runs. Smoluchowski approximation was used to calculate zeta potential from the electrophoretic mobility. All measurements were performed in triplicate at 25°C (49).

### 2.7.3 DSC analysis

DSC analysis of developed albumin nanoparticles were carried out using a Differential Scanning Calorimeter (DSC-60, Shimadzu, Japan) at a heating rate of 10°C per minute in the range of 30°C to 250°C under inert nitrogen atmosphere at a flow rate of 40 ml/min (50).

### 2.7.4 FTIR analysis

FTIR spectrum of developed albumin nanoparticles were measured with a FTIR spectrophotometer (IR Affinity -1S (Shimadzu, Japan) in range 400–4000 $cm^{-1}$ using a resolution of 4 $cm^{-1}$ (50).

### 2.7.5 XRD analysis

X-ray diffraction patterns of developed albumin nanoparticles were obtained using X-ray diffractometer (RigakuUltima IV; Japan) in which Cu-Kα line used as a source of radiation by operating at the voltage 40 kV and the current applied was 40 mA. Both samples were measured in the 2θ angle range between 5°-50° with a scanning rate of 3°/min and a step size of 0.02° (50)

### 2.7.6 Morphology

Morphology of developed albumin nanoparticles were observed using transmission electron microscope (Philips CM200) (51).

## 2.8 Evaluation of developed albumin nanoparticles

### 2.8.1 Estimation of entrapment efficiency and drug loading

TMZ and LND entrapment in the TNPs, HA-TNPs, CS-TNPs, LNPs and Lf-LNPs respectively were determined indirectly by measuring the amount of free TMZ and LND in the supernatant using UV–visible spectrophotometer (Shimadzu UV-1700) (50). Then percentage entrapment efficiency (% EE) and drug loading (% DL) was determined using the formula:

$$\% EE = \frac{(\text{Total drug} - \text{Free drug})}{\text{Total drug}} \times 100 \text{ .....Equation 1}$$

$$\% DL = \frac{\text{Entrapped drug}}{\text{Total weight of nanoparticles}} \times 100 \text{.....Equation 2}$$

### 2.8.2 *In vitro* drug release

The *in-vitro* drug release studies were carried out using dialysis bag method at 37 °C under mild stirring (50 rpm) (52). Pure drug (TMZ and LND) and drug loaded albumin nanoparticles (TNPs, HA-TNPs, CS-TNPs, LNPs and Lf-LNPs) (equivalent to 5 mg drug) were taken into dialysis bags (MWCO = 12000), and were immersed into beakers containing 30 ml of release medium. At predetermined period, 1.0 ml of sample was withdrawn and same quantity of fresh buffer was added into the beaker to maintain sink condition. The amount of TMZ and LND released was determined using UV spectrophotometer and spectrofluorophotometer respectively.

### 2.8.3 Bio-Interactions

#### 2.8.3.1 Interactions with plasma proteins

The interactions of developed albumin nanoparticles with plasma protein were studied to assess the bio-stability of the prepared nanoparticles. Nanoparticles in concentration of 5 mg/ml were dispersed in PBS (pH 7.4) and mixed with protein solution (10% w/v) respectively and incubated at 37°C in orbital shaker. After 4 h, the mixture was centrifuged at 12000 rpm and obtained pellet was redispersed in double distilled water. Then particle size and zeta potential of nanoparticles dispersion were measured (53,54).

#### 2.8.3.2 Interactions with cell culture media (DMEM)

The interactions of developed albumin nanoparticles with DMEM media were studied by dispersing nanoparticles (5 mg/ml) in PBS (pH 7.4) and mixed with DMEM media respectively and incubated at 37°C in orbital shaker. After 4 h, the mixture was centrifuged at 12000 rpm and obtained pellet was redispersed in double distilled water. Then particle size and zeta potential of nanoparticles dispersion were measured (39,53).

#### 2.8.3.3 Interactions with serum

The interactions of developed albumin nanoparticles with serum were studied by dispersing nanoparticles (5 mg/ml) in PBS (pH 7.4) and mixed with 50% v/v serum respectively and incubated at 37°C in orbital shaker. After 4 h, the mixture was centrifuged at 12000 rpm and obtained pellet was redispersed in double distilled water. Then particle size and zeta potential of nanoparticles dispersion were measured (54).

**2.8.3.4 Haemolysis study**

For haemolysis study, 1.0 ml blood sample was collected in EDTA solution (30 µl) containing eppendorf tube from the Sprague Dawley rat by retro-orbital puncture. Blood sample was then centrifuged at 5000 rpm for 10 min at 4 °C to separate the red blood cells (RBCs). The separated RBC pellet was re-suspended in normal saline and plasma components were removed by washing with normal saline (0.9 % w/w Sodium Chloride in water) 3 times before use. Then 0.5 % v/v RBCs were prepared by re-suspending RBC pellet (250 µl) in 50 ml of normal saline. Then 1 ml of RBCs was added to plain drug suspension and developed albumin nanoparticles 1mg equivalent amount of drug dispersed in 1ml of saline. For positive and negative control, 2.0% Triton-X100 (1ml) and 0.5% DMSO was used respectively. After treatment (with drug suspension, nanoparticles, positive control and negative control), RBC dispersion was gently stirred to uniformly disperse RBCs. The treated dispersions were stored at 37°C for 30 min in incubator. After incubation, all the samples were centrifuged at 3000 rpm for 12 min at 4 °C to separate the RBC mass and the solutions were analyzed for UV absorbance at λmax of 540 nm against normal saline as a reference solution (55,56). Percentage of haemolysis was determined using following equation:

$$\%\, Haemolysis = \left[\frac{A_{540}\ of\ sample - A_{540}\ of\ negative\ control}{A_{540}\ of\ positive\ control - A_{540}\ of\ negative\ control}\right] \times 100 \quad \text{……Equation 3}$$

**2.9 *In vitro* cell line studies**

**2.9.1 *In vitro* BBB Passage Study**

The permeation of drug (TMZ and LND) and developed albumin nanoparticles across the BBB was assessed by *in vitro* BBB model (co-culture model) (50). After development of co-culture model, 1 ml of drug and various developed albumin nanoparticles at drug concentration of 2 mg/ml was added to the luminal compartment of inserts. Then, 200 µl of medium was

withdrawn from the basal compartment at predetermined time interval (0, 2, 24 and 48 h), replacing with fresh medium. The permeation of drug through the *in vitro* BBB model was determined by HPLC and spectrofluorophotometer for TMZ and LND respectively (50) and transport ratio of drug and nanoparticles was determined using the following equation:

$$Transportatio\ (\%) = \left(\frac{Wn}{W}\right) * 100 \quad \text{.....Equation 4}$$

Where, Wn = amount of drug in the basal chamber at time "n" (n = 2, 24 and 48 h); W = amount of drug added in the apical chamber.

### 2.9.2 *In-vitro* cell viability assay

The *in-vitro* cell cytotoxicity of the prepared nanoparticles along with pure drug against U-87 MG cells was estimated by MTT assay. Cells were incubated with different concentrations (10-50 µg/ml) of pure drug and developed albumin nanoparticles for 24 and 48 h, respectively and cytotoxicity was determined using MTT reagent (39).

### 2.9.3 Cell cycle analysis

U87 MG cells (density of $1\times10^5$ cells/well) were seeded on plates and incubated at 37 °C for 24 h. After 24 h, old medium was discarded, replaced with fresh medium containing TMZ, TNPs, HA-TNPs and CS-TNPs at concentration equivalent to 100 µg/ml of pure drug and incubated for another 24 h. Then cells were detached using trypsin-EDTA, washed with PBS and fixed using 70% ethanol. Staining of the fixed cells were done with 0.5 ml of PBS containing 0.5 µg/ml propidium iodide, 10 µg/ml RNase A and 0.1% triton X-100. The cells were incubated for 30 min at room temperature in dark and cell cycle analysis was performed using BD FACS Aria III (BD Biosciences, CA). Data was analyzed using BD FACS Diva software version 6.1.3 (57).

### 2.9.4 Cellular uptake and uptake mechanism

The uptake poisoning method was utilized to investigate the cellular uptake and endocytosis mechanism of drug and developed albumin nanoparticles in U-87 MG cell monolayers. U-87 MG cells ($1\times10^5$ cells/well) were seeded on 6-well plate and allowed to grow for 24 h. After 24h, old media was replaced with fresh culture medium containing sodium azide (0.1 % w/v), chlorpromazine (10 µg/ml) and nystatin (50 µg/ml) and cell monolayers were incubated for 1 h at

37 °C. After 1 h, various formulations (25 µg/ml each) were added and co-incubated for 4 h. After incubation, cells were lysed using 1% Triton X-100 and amount of drug inside the cells was analyzed (39,58).

### 2.9.5 CD44 receptor targeting assay

CD44 blocking assay was used to study uptake of HA-TNPs and CS-TNPs by CD44 receptor. For that, the cells were pretreated with 10 mg/ml free HA polymer (175-350 kDa and hydrated overnight in serum and antibiotic free medium) for 1 h before addition of HA-TNPs and CS-TNPs, again incubated for an additional 12 h and 24h. After incubation, cells were lysed using 1% Triton X-100 and amount of TMZ inside the cells was analyzed by HPLC (39).

### 2.9.6 Lactoferrin (Lf) receptor targeting assay

Lf receptor blocking assay was used to study uptake of Lf-LNPs by Lf receptor. For that, the cells were pretreated with Lf (100 µg/ml) for 30 min followed by washing and treatment with FITC conjugated Lf-LNPs, again incubated for an additional 4h. After incubation, cells were lysed using 1% Triton X-100 and amount of FITC inside the cells was analyzed by spectrofluorophotometer (58) .

### 2.9.7 Reactive oxygen species (ROS) generation study

Dichlorofluorescein (DCF) assay was utilized to estimate ROS generation in U-87 MG cells. The cells were seeded in 96 well plate and incubated for 24 h. After 24h, medium was replaced with fresh medium containing drug (TMZ and LND) and developed albumin nanoparticles (10-50 µg/ml). In this analysis, 10 µg $ml^{-1}$ of $H_2O_2$ was used as a positive control while untreated cells were used as negative control. Cells were harvested, washed once with PBS and incubated with 10 µM $H_2$DCFDA (2,7-dichlorodihydrofluorescein diacetate) (in PBS 1×) for 30 min at 37°C prior to analysis. The DCF fluorescence was then recorded at 535 nm using a plate reader (Fluoroskan Ascent CF (Labsystems, USA)). The generated ROS was expressed as a ratio of the fluorescence of DCF of treated cells to that of untreated cells (39).

### 2.9.8 Scratch assay

U-87 MG cells ($2 \times 10^5$ cells/ well) were plated in 6-well plates and incubated at 37°C and 5% $CO_2$ environment to form a confluent monolayer. After monolayer formation, scratch was created with the help of p200 pipette tip. The scratch containing cells were washed with growth medium to remove the debris and smoothening of scratch. Then old media was replaced with 1 ml of fresh medium specific for the in vitro scratch assay. To obtain the same field during the image acquisition, markings were created to be used as reference points close to the scratch. Then cells were treated with drug (TMZ and LND) and various developed albumin nanoparticles at a concentration equivalent to 25 µg/ml of drug. In control group wells, no sample was added. Then plates were incubated at 37 °C and examined daily until the scratch in the control group wells was filled completely with cells (59)

**2.10 In vivo studies**

All experimental protocol for *in vivo* studies were reviewed and approved by Institutional Animal Ethics Committee, Pharmacy department, The M. S. University of Baroda, Vadodara, vide protocol approval no: MSU/IAEC/2018-19/1830. All the experimental procedures were carried out as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines released by Ministry of Environment, Forests and Climate Change and Ministry of Social Justice and Empowerment, Government of India, New Delhi, India. All the *in vivo* experiments were performed on Wistar rats. Wistar rats (any sex) of 150-200 g were obtained from official CPCSEA breeder. The rats were housed in cages placed in an animal room with a constant temperature of 22 °C and a fixed 12-hour light-dark cycle. All animals were handled and housed according to the guidelines and manual set by the Committee of the Care and Use of Laboratory Animals. The rats were given standard rat chow ad libitum and water. After acclimatization the studies were performed as follows:

**2.10.1 Pharmacokinetic study**

Pharmacokinetic study on Wistar rats were performed to obtain plasma concentration-time profile of TMZ, TNPs, HA-TNPs and CS-TNPs. Thirty Wistar rats (150–200 g) were randomly divided into five groups (n=6) and were fasted over night before the experiment but allowed to drink water freely. Animals in group I were control, animal in group II received pure TMZ dispersion, the animals in group III received TNPs, animals in group IV received HA-TNPs and

animals in group V received CS-TNPs by intravenous route via tail vein at a dose of 3 mg/kg of TMZ (60). At predetermined intervals (0.5h, 1h, 3h, 6h, 12h, 24h, 48h, 60h and 72h), blood samples were collected retro orbitally in EDTA containing tubes and centrifuged at 5000 rpm for 20 min by a cooling centrifuge (Remi Equipments Ltd). The separated plasma was stored at -20 °C until further analysis (50).

**Parameters:**

- Plasma concentration versus time was plotted and pharmacokinetic parameters were determined after dose normalization.
- $C_{max}$ was directly determined from the concentration–time profile.
- Mean residence time (MRT) and elimination rate constant ($K_{el}$) for the drug were determined from the plot.
- Area under the curve (AUC) was calculated by the trapezoidal rule

### 2.10.2 Biodistribution study

Wistar rats were randomly divided into five groups with six animals for each time point. Rats in group I received saline solution (control group), group II received pure TMZ dispersion, animals in group III, IV and V received TNPs, HA-TNPs and CS-TNPs at a dose of 3 mg/kg of TMZ via tail vein. At predetermined time intervals (12h and 24h), rats were sacrificed by euthanization and different organs like brain, heart, lung, liver, kidney and spleen were isolated and blood was collected. Blood samples were processed as mentioned earlier in pharmacokinetic study. Organs were rinsed and homogenized with cold saline by tissue homogenizer, centrifuged (5000 rpm × 20 min) at 4°C and stored at -20 °C in till further analysis. The TMZ level in plasma and different organs was determined by HPLC analysis (61).

### 2.10.3 In vivo toxicity study

Rats were randomly divided into five groups (n=6 in each group). Saline solution, pure TMZ, TNPs, HA-TNPs and CS-TNPs at a dose equivalent to 30 mg/kg were administered intravenously via tail vein to different group animals. After dose administration, all the animals were maintained on water and normal diet. After 7 days, blood was collected retro-orbitally and

subjected to biochemical parameter estimation viz. blood parameters and clinical parameters using standard kits through standard procedures in an automated bioanalyser (61,62).

### 2.10.4 *In vivo* anticancer activity of Lf-LNPs

Balb/c mice (8-10 weeks) were used for the experiment (63,64). Briefly, U87 MG cells (cell density = $1\times10^5$) were injected on the back of the mice and allowed to form tumors. The animals were divided into three groups (n = 3): Group I (model control; untreated group), Group II (Standard control; LND) and Group III (Lf-LNPs). After reaching the tumor a palpable size (100 ± 10 mm), 0.9 % saline solution, pure LND (3 mg/kg) and Lf-LNPs equivalent to 3 mg/kg of LND were administered to group I, group II and group III respectively via i.v. route once a week. Tumor volume was measured using digital vernier calipers (Mitutoyo JAPAN). Tumor volume was calculated by the below mentioned equation:

$$Volume = \frac{Width^2 \times Length}{2} \quad \text{.....Equation 5}$$

At the end of the experiment the animals were sacrificed by overdose of thiopentone sodium. The animals were dissected and tumors were excised. The excised tumors were immediately imaged.

### 2.11 Stability studies

The stability of the lyophilized developed albumin nanoparticles were investigated by storing samples at refrigerated condition (4°C) and at room temperature (25°C ±2°C) for 3 months. At regular time interval of 1 month, samples were withdrawn and redispersed in saline solution to check particle size, assay and zeta potential (65,66) .

### 2.12 Statistical Data Analysis

Results are given as mean ± SD. Statistical significance was tested by two-tailed Student's t test or one-way ANOVA. Statistical significance was set at $P < 0.05$.

**3.0 Key findings**

Firstly temozolomide loaded nanoparticles (TNPs) were developed and optimized. TNPs were prepared by modified desolvation method. TNPs were preliminary optimized by OVAT design to identify the critical parameters and their working range for further optimization. During preliminary optimization, various parameters like polymer to drug ratio, polymer concentration, organic phase volume, crosslinker (gluteraldehyde) concentration, rate of addition of organic phase, effect of stirring speed, effect of pH, etc were screened. The results indicated that polymer to drug ratio ratio, organic phase volume and crosslinker concentration had significant effect on quality attributes (particle size, PDI, zeta potential and entrapment efficiency) of nanoparticles. So these parameters were further optimized using Box-Behnken response surface methodology and results indicated that all three parameters had significant effect on particle size and entrapment efficiency of TNPs while non significant effect on PDI and zeta potential. In case of particle size, GA concentration showed most significant effect as compared to polymer: drug and organic phase volume on particle size. In case of entrapment efficiency, polymer: drug ratio and interaction of polymer: drug ratio and organic phase volume was having most significant effect as compared to GA concentration and organic phase volume.

After optimization of TNPs, surface modification was done with hyaluronic acid (HA) and chondroitin sulphate (CS) using carbodiimide chemistry to achieve CD44 mediated targeted delivery of TNPs (HA-TNPs and CS-TNPs). Surface modification with HA and CS was optimized on the basis of particle size, PDI, zeta potential and conjugation efficiency. Different parameters like molecular weight of HA, ligand to NPs ratio and stirring time was selected as parameter of optimization. Conjugation of HA and CS with TNPs was also confirmed by FTIR studies. For HA-TNPs development, low molecular weight HA, HA: NPs (1:1) and 30 min of stirring was found to be optimum while in case of CS-TNPs, CS: NPs (1:1) and 12 h stirring was found to be optimum to get the highest conjugation efficiency of 78.34 % ± 1.91 % and 74.34 % ± 1.87 % respectively.

In the next step, surface modified HA-TNPs and CS-TNPs along with TNPs were characterized and evaluated on the basis of various in vitro studies. The dynamic light scattering results indicated that hydrodynamic diameter of TNPs was within the range of 150-160 nm while

in case of HA-TNPs and CS-TNPs it was increased and was in range of 200-350 nm. The increase in the hydrodynamic diameter of surface modified nanoparticles may be attributed to targeting moiety. The particle size distribution pattern indicated uniformity of nanoparticles dispersion which made them suitable for intravenous administration. Zeta potential results indicated the stability of the developed nanoparticles.

DSC study indicated that in both the cases (TNPs and surface modified TNPs) drug was molecularly dispersed in the carrier and totally encapsulated within the nanoparticles as the exothermic peak of drug was not present in the final formulations.

FTIR data also confirmed the presence of drug in the nanoparticles as all the characteristic peaks of drug was present in the developed nanoparticles with lesser intensity. FTIR data also confirmed the successful conjugation of HA and CS with TNPs as the new amide bond peak was observed in both HA-TNPs and CS-TNPs.

XRD spectrum revealed conversion of crystalline nature of TMZ in amorphous nature after encapsulation in nanoparticles as the highly intense characteristic peaks of drug was converted in the lesser intensity peaks.

TEM image of HA-TNPs and CS-TNPs showed roughly spherical shape, exhibiting a dark core surrounded by a lighter gray rim likely corresponding to the HA and CS conjugation respectively. Size of HA-TNPs and CS-TNPs obtain by TEM was lesser than obtained by zetasizer (DLS measurement). This may be due to presence of water surrounding HA-TNPs during DLS in comparison with size measured in dried state by TEM.

In vitro release data revealed biphasic release of TMZ from TNPs, HA-TNPs and CS-TNPs. All the developed nanoparticles showed initial burst release than slower and sustained release. The surface modified HA-TNPs and CS-TNPs showed more sustained release pattern of drug as compare to TNPs this may be due to slower diffusion of drug from additional HA and CS layer over the TNPs.

Results of cell viability assay of different developed nanoparticles showed higher suppression of cells as compared to pure TMZ. Surface modified TNPs (HA-TNPs and CS-TNPs both) showed higher cell suppression than TNPs. This may be due to higher uptake of the

nanoparticles because of presence of targeting moiety over the surface which enhanced the cellular uptake of nanoparticles by binding with the CD44 receptor present in the surface of tumor cells.

In vitro BBB passage study also confirmed the higher permeation of nanoparticles through BBB than pure drug. This may be due to smaller size of nanoparticles than the pure TMZ. As compared to TNPs, HA-TNPs and CS-TNPs showed higher permeation this was due to the fact that CS and HA also plays role in crossing BBB. The sequence of BBB passage was as follows: HA-TNPs > CS-TNPs > TNPs > TMZ

Effect of developed nanoparticles on cell cycle arrest was also carried out and results demonstrated that as compare to pure TMZ, CS-TNPs showed higher G2/M phase cell arrest. That suggested higher inhibition of growth of cancerous cells. In case of HA-TNPs no significant changes as compare to non treated cells were observed.

Surface modification plays important role in cellular uptake of nanoparticles. HA and CS both are CD44 receptor targeting moieties. CD44 receptors are over expressed in brain tumors. To achieve the brain tumor specific targeting these CD44 receptors were targeted by HA and CS. The cellular uptake results indicated that as compared to pure drug and TNPs, cellular uptake of surface modified nanoparticles were higher in both HA-TNPs and CS-TNPs. All the developed nanoparticles were taken up by caveolae mediated pathway. CD44 receptors were also involved in the uptake mechanism and this was confirmed by the CD44 receptor blocking assay.

ROS generation in U-87 MG cells was estimated and the results demonstrated concentration dependent ROS generation. As the concentration of TNPs, HA-TNPs and CS-TNPs increased, an increase in ROS generation was also observed. This may be correlated with the fact that increase in concentration of TNPs, HA-TNPs and CS-TNPs led to increased concentration of released TMZ leading to increase in ROS generation. HA-TNPs and CS-TNPs showed higher ROS generation as compared to pure TMZ and TNPs. This may be due to higher uptake of HA-TNPs and CS-TNPs via CD44 receptor which led to increased TMZ concentration in the cells that ultimately caused increased ROS generation.

The results of scratch assay indicated slower cell migration as compared to control group after treated with TMZ and developed nanoparticles. As compared to TMZ, TNPs and surface modified TNPs (HA-TNPs and CS-TNPs) indicated significant restriction of cell migration.

In vivo pharmacokinetic and biodistribution studies revealed improvement in pharmacokinetic profile and therapeutic concentration of TMZ in brain after encapsulating it in nanoparticles.

The quantitative biodistribution study of TMZ, TNPs, HA-TNPs and CS-TNPs was performed to assess the passage of TMZ through the BBB and its distribution in various vital organs viz. brain, liver, heart, lungs, kidney and spleen. The distribution of TMZ in brain tissues was approximately six folds, eight folds and nine folds higher with TNPs, HA-TNPs and CS-TNPs respectively as compared to free TMZ. This may be due to smaller size of nanoparticles as compare to pure drug and enhanced BBB permeability. As mentioned earlier HA and CS also plays role to enhance BBB permeation, presence of HA and CS over the nanoparticles facilitate the BBB crossing which led to enhance concentration of drug in the brain. The obtained results showed correlation with in vitro BBB permeation study.

Further, the results revealed significant reduction in the distribution of drug to highly perfused organs when given as nanoparticles. This may be attributed to surface modification of nanoparticles that led to prevented opsonization. After 24 h, distribution of TMZ released from HA-TNPs and CS-TNPs were more or less similar in spleen and heart whereas in liver and lungs, distribution was significantly decreased as compared to pure TMZ. Distribution of CS-TNPs was high in kidney as compared to free TMZ may be due to presence of hydrophilic polymer over the surface of TNPs

Furthermore the biodistribution and toxicity data suggested safety of developed nanoparticles as accumulation of TMZ in other vital organs decreased and no significant changes were seen in biochemical parameters of rats treated with formulations.

The interaction of synthesized nanoparticles was also assessed with respect to blood and culture media. No significant interactions were observed.

The results of stability studies indicated no significant change in particle size, % assay and zeta potential of prepared nanoparticles at both refrigerated condition and room temperature for three months which indicated their stability.

Lenalidomide loaded albumin nanoparticles (LNPs) were also prepared by desolvation method and optimized by OVAT design. Then according to optimized formula scale up batch (4X) was prepared and subjected to lyophilization.

After optimization of LNPs, surface modification was done using lactoferrin (Lf) to achieve lactoferrin receptor targeting. As LNPs has negative surface charge and Lf has positive surface charge at neutral to slight alkaline medium, Lf is physically coated over the LNPs. For the optimization of Lf coating two different parameters like Lf concentration and time of stirring was considered and optimization was done with placebo nanoparticles on the basis of particle size and zeta potential measurement. The results indicated that 1% w/v Lf and 1 hr stirring was sufficient to coated LNPs for achieving desired particle size and zeta potential. Confirmation of the coating was done on the basis of zeta potential. Initially zeta potential of LNPs was negative (-16.7 ± 2.9 mV) which was shifted to positive side (0.9 ± 0.5 mV) after Lf coating. Percentage coating and presence of Lf was also estimated by Bradford protein estimation assay which indicated approximate 31.78 % ± 2.3 % coating of Lf with the LNPs. To increase the percentage of Lf coating higher concentration of Lf also tried but particle size was significantly enhanced so for preparing final optimized batch of Lf coated LNPs (LF-LNPs) 1% w/v Lf and 1hr stirring was used. After that scale up batch was prepared and lyophilized.

Surface modified Lf-LNPs along with LNPs were further characterized and evaluated on the basis of various in vitro studies. The dynamic light scattering results indicated that hydrodynamic diameter of LNPs was within the range of 120-130 nm while in case of Lf-LNPs it was increased and was in range of 145-150 nm. The increase in the hydrodynamic diameter of surface modified nanoparticles may be attributed to Lf coating. The particle size distribution pattern indicated uniformity of nanoparticles dispersion which made them suitable for intravenous administration. Zeta potential results indicated the successful coating and stability of the developed nanoparticles.

Entrapment efficiency of LND in LNPs and Lf-LNPs was measured by free drug estimation and entrapment was found to be 92.96 % ± 2.37 % and 90.05 % ± 2.87 % respectively while drug loading was found to be 12.13 % ± 1.77 % and 11.15 % ± 1.17 % respectively.

FTIR data also confirmed the presence of drug in the nanoparticles as all the characteristic peaks of LND was present in the developed nanoparticles with lesser intensity. FTIR data also confirmed the successful coating of Lf over LNPs.

XRD spectrum revealed conversion of crystalline nature of LND in slightly amorphous nature after encapsulation in nanoparticles as the highly intense characteristic peaks of drug was converted in the lesser intensity peaks and also confirmed presence of LND in the developed formulation.

TEM image of Lf-LNPs showed spherical shape, exhibiting a dark core surrounded by a lighter gray rim likely corresponding to the Lf coating. Size of Lf-LNPs obtain by TEM was lesser than obtained by zetasizer (DLS measurement). As mentioned earlier, this may be due to presence of water surrounding Lf-LNPs during DLS in comparison with size measured in dried state by TEM as mentioned earlier.

In vitro release data revealed biphasic release of LND from LNPs and Lf-LNPs in which initial burst release observed and after that slower and sustained release was observed. The initial fast release may be due to release of surface associated drug while sustained release may be due to slower diffusion of drug from nanoparticles.

Results of cell viability assay of developed nanoparticles showed higher suppression of cells as compare to pure LND. Surface modified Lf-LNPs showed higher cell suppression than LNPs. This may be due to higher uptake of the nanoparticles because of presence of targeting moiety over the surface which enhanced the cellular uptake of nanoparticles.

In vitro BBB passage study also confirmed the higher permeation of nanoparticles through BBB than pure drug. This may be due to smaller size of nanoparticles than the pure LND. As compared to LNPs, Lf-LNPs showed higher permeation this was due to Lf receptor mediated targeting as Lf receptors are over expressed in BBB. The sequence of BBB passage was as follows: Lf-LNPs > Lf-PNPs > LNPs > PNPs > LND.

Surface modification plays important role in cellular uptake of nanoparticles. Lf receptors are over expressed in BBB as well as brain tumor cells so Lf receptor mediated targeting strategy was utilized the deliver the drug (LND) to the target site by coating LNPs with Lf. For confirming the Lf receptor mediated targeting, the cellular uptake study was performed and the cellular uptake results indicated that as compared to pure drug and LNPs, cellular uptake of surface modified nanoparticles (Lf-LNPs) was higher. LNPs were taken up by caveolae mediated pathway while uptake of Lf-LNPs was clathrin mediated. Lf receptors were also involved in the uptake mechanism and this was confirmed by the Lf receptor binding assay.

ROS generation in U-87 MG cells was estimated and the results demonstrated concentration dependent ROS generation. As the concentration of LNPs and Lf-LNPs increased, an increase in ROS generation was also observed. This may be correlated with the fact that increase in concentration of LNPs and Lf-LNPs led to increased concentration of released LND leading to increase in ROS generation. Lf-LNPs showed higher ROS generation as compared to pure LND and LNPs. This may be due to higher uptake of Lf-LNPs via Lf receptor which led to increased LND concentration in the cells that ultimately caused increased ROS generation.

The results of scratch assay indicated slower cell migration as compared to control group after treated with LND and developed nanoparticles. As compared to LND and LNPs and Lf-LNPs indicated significant restriction of cell migration.

The interaction of synthesized nanoparticles was also assessed with respect to blood and culture media. No significant interactions were observed.

After getting satisfactory results from in-vitro characterization and evaluation, Lf-LNPs were subjected to in vivo tumor regression study for assess the anticancer potential of developed formulation. The results indicated that as compared to untreated model control group and standard control (LND treated), lesser tumor growth was observed in Lf-LNPs treated animals which indicated better anticancer activity of LND encapsulated in the NPs.

The results of stability studies indicated no significant change in particle size, % assay and zeta potential of prepared nanoparticles at both refrigerated condition and room temperature for three months which indicated their stability.

**4.0 Conclusion**

In the present work, hyaluronic acid conjugated, chondroitin sulphate conjugated and lactoferrin coated albumin nanoparticles were developed and optimized for CD44 receptor mediated and lactoferrin receptor targeted delivery of temozolomide and lenalidomide respectively. The developed nanoparticles were characterized and evaluated for various physicochemical properties like particles size, PDI, zeta potential, morphology, drug release, stability etc. In vitro release profile demonstrated sustained release of both the drugs after encapsulating in the nanocarrier. In vitro cell line studies demonstrated that developed nanoparticles were able to cross blood brain barrier and reache to target tumor site by caveolae mediated endocytosis pathway. Hyaluronic acid and chondroitin sulphate enhanced the cellular uptake of TNPs (HA-TNPs and CS-TNPs) by CD44 receptor mediated delivery while lactoferrin enhanced the cellular uptake of LNPs (Lf-LNPs) by Lf receptor mediated delivery and enhanced the therapeutic activity and performance of temozolomide and lenalidomide towards cancerous cells as compared to pure drug. The results obtained by *in vivo* studies were also correlated with findings of *in vitro* studies. Pharmacokinetic studies proved the enhanced concentration of temozolomide in brain when delivered by nanoparticles and toxicity data indicated safety of developed nanoparticles as no significant changes were seen in biochemical parameters of treated rats. In case of lenalidomide, results of in vivo tumor regression study suggested that Lf-LNPs showed better anticancer activity than standard control.

In conclusion, the results of this study suggested that the developed hyaluronic acid conjugated and chondroitin sulphate conjugated albumin nanoparticles loaded with temozolomide (HA-TNPs and CS-TNPs) and lactoferrin coated albumin nanoparticles (Lf-LNPs) loaded with lenalidomide have potential to target brain tumor and reduce toxicity towards normal cells. The finding suggest that the efficient brain tumor targeting with maximum safety and efficacy and minimum toxicity can be achieved for effective treatment of brain tumors by employing surface modified albumin nanoparticles as delivery platform.

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