Details of the research work

Background and Gaps in existing research:

Glioblastoma is an abnormal proliferation of the glial cells and has the highest mortality rate. Among the few drugs like carmustine, lomustine, everolimus, bevacizumab, and temozolomide approved for the treatment of glioblastoma, temozolomide (TMZ) is considered a first choice. TMZ is a small molecule amphiphilic in nature. It attains 100 % bioavailability when administered in the form of oral capsules and intravenous infusion. Despite the high permeability through the blood-brain barrier, only 20-30 % brain bioavailability is achieved. This prodrug undergoes non-enzymatic modification at plasma pH. The resulting active metabolites cannot cross the blood-brain barrier due to their hydrophilic nature. The short half-life of TMZ limits the therapeutic efficacy. A higher dose of the drug needs to be administered, which also leads to myelosuppression, thrombocytopenia, or neutropenia. Moreover, current temozolomide therapy involves multiple dosing [1–3]. The limitations of conventional therapies can be tackled with nanomedicine.

Nanoparticulate systems for TMZ using phospholipids, lipids, polymers or any other form can provide controlled release, longer circulation time, enhance plasma concentrations and improve its concentration in the brain. Enhanced concentration in brain and better therapeutic efficacy may result in reduction of the dose, lower toxic or side effects and improved patient compliance. Although various nanoformulations like polymeric nanoparticles, solid lipid nanoparticles, nanostructured lipid carriers, and liposomes have been investigated [4,5], complex composition, sophisticated process, and poor entrapment of TMZ within these nanoparticles remain a challenging task. In the course of time, nanoproducts gained significant importance but they had to face numerous challenges before reaching the market such as complex preparation techniques involving multiple steps, stability issues, reproducibility, large scale manufacturing, and regulatory barriers. Most of the manufacturing methods used for the preparation of the nanoformulations are feasible only at the lab scale, prepared by time-consuming and costly processes, utilize organic solvents, and are difficult to scale up. All of these, limit the translation of nanotechnologies for clinical application. It is a challenge to scale-up nanoformulations from lab scale to industrial scale maintaining the necessary properties of the designed nanoparticles [6,7]. Therefore, information

related to various factors causing variability needs to be adequately understood by investigating the manufacturing process in detail throughout the development process. Several material or process parameters affecting the final characteristics of the product need to be identified. The relationship between input variables (material/ process) and nanocarrier characteristics is complicated and an elaborate investigation of the effect of individual variable is essential to ensure the successful scale-up.

Objective:

Based on the existing gap and challenges of delivery of TMZ for effective and long term therapy with better patient compliance, it was planned to design and formulate TMZ-loaded lipid-based nanoparticulate systems of liposomes and lyotropic liquid crystals. These could prolong the plasma circulation time of TMZ, provide selective and enhanced delivery of the drug to the brain with reduced side effects and improve the efficacy of the treatment.

Lipid-based nanocarriers have gained much attention due to their biocompatible nature. Liposomes are one of the nanocarriers successfully entering the market. These are phospholipid vesicles enclosing an aqueous core. Advanced research led to the discovery of unique structures known as lyotropic liquid crystals (LLCs). This mesophase contains properties of a crystal and liquid simultaneously. Liposomes and Lyotropic liquid crystals are nanocarriers were selected to be investigated as these have shown great potential to entrap amphiphilic drugs (contain both hydrophilic as well as lipophilic regions). In order to achieve longer plasma circulation, it was planned to coat nanocarriers with a polymer. DSPE-PEG 2000 is a non-ionic polymer, soluble in aqueous and organic medium, biocompatible, biodegradable, and non-toxic. It is expected to modify the surface energies of the nanocarrier, prevent their macrophage uptake/opsonization and hence prolong the plasma circulation time further.

Quality by design is an important tool which helps gain process understanding, reduce chances of failure in later stages of product development and help shorten the translation process. Hence, it was planned to optimize the selected lipid based nanocarriers using the principles of Quality by Design which would include investigating the effect of various critical material attributes, and process parameters on the critical quality attributes of lipid based nanocarriers.

A method of preparation that does not require organic solvent, involves minimum number of steps, which is highly reproducible and industrial feasible was planned to be selected.

Methodology:

Two lipid-based nanocarriers, liposomes, and lyotropic liquid crystals were selected for investigation. A quality target product profile was designed. Critical quality attributes, material attributes, and process parameters were identified using the principles of Quality by Design. The effect of various material attributes and process parameters on critical quality attributes were investigated in detail [8,9]. The designed nanocarriers were characterized for particle size, size distribution, zeta potential (using Zetasizer), morphology (using microscopic techniques), entrapment efficiency (by dialysis bag technique), reproducibility, drug release (using dialysis bag), hemolysis, cell cytotoxicity, cell uptake, pharmacokinetics, and biodistribution in rats [5,10].

Results and discussion:

During the formulation of liposomes and lyotropic liquid crystals (LLCs), various lipids, methods of preparation, and size reduction techniques were screened. Membrane extrusion technique was found to produce liposomes with smaller particle sizes in a reproducible manner. From the various phospholipids screened, synthetic phospholipids were found to provide maximum TMZ entrapment. For LLCs, hot melt emulsification followed by probe sonication was found to provide a smaller particle size.

Initially, the Quality Target Product Profile (QTPP) was designed and the various critical quality attributes (CQAs) were derived as summarized in **Table 1 and 2.**

Table 1: Quality Target Product Profile of TMZ loaded Nanocarriers

Quality attribute	Target	Justification
Dosage design	PEGylated Liposomes/ PEGylated Lyotropic Liquid Crystals (LLCs)	Liposomes and LLCs are biocompatible and are suitable for entrapping amphiphilic drugs. These release the drug in a prolonged manner thus helping to improve in-vivo circulation time. PEGylation helps improve the plasma circulation time.

Dosage form	Lyophilized powder to be reconstituted	Ensures stability of drug during storage
Route of administration	Intravenous	Pharmaceutical equivalence: Same route as Reference Listed Drug (RLD)
Physical attributes	White powder	-
Assay	95-115 %	To ensure the therapeutic effectiveness
pH	Near to plasma pH (4-6)	Required to prevent drug degradation, maintain isotonicity, and prevent hemolysis
Osmolality	290±20 mOsm/kg	To maintain blood osmolarity and prevent hemolysis
Biocompatibility	Non-hemolytic and non-toxic	To prevent hemolysis and reduce toxicity
Particle size	80-150 nm	Recommended to prolong plasma circulation time and reduce clearance rate
Zeta potential	Near to neutral	Avoids protein binding and uptake by organs like the liver and lungs
% Entrapment efficiency	Maximum	To obtain maximum therapeutic efficiency
% Drug loading	Optimum	Affects drug release patterns, patient compliance, and the cost of the product
Drug release	Prolonged release compared to free drug	Provide longer plasma circulation time of drug and improved brain bioavailability
Pharmacokinetics	Longer circulation time, higher brain bioavailability and reduced clearance rate in comparison to free drug.	Required to achieve an improved therapeutic effect and patient compliance
Stability	No visible signs of instability during preparation and storage	Ensures stability of drug during shelf-life and required for marketing approval
Storage	Store between 2^{o}C to 8^{o}C	Required to ensure the stability of the drug and the lipid carrier
Container closure system	Air-tight glass vial	Ensure protection of drug and the excipients

Administration/ Labeling	Reconstitute with sterile water for injection/ Normal saline before administration Store at room temperature and use within 14 hours	Required to ensure the stability of the drug and therapeutic use

Table 2: Critical Quality Attributes of TMZ loaded Nanocarriers

Quality Target Product Profile (QTPP)	Impact analysis	Severity analysis	Critical or not?	Justification
Dosage design	No	Yes (But is Already decided)	No	-
Dosage form	No	No (Is already decided)	No	-
Route of administration	No	Yes (But is Already decided)	No	-
Dosage strength	Yes	Yes	Yes	Affects the therapeutic effectiveness
Physical attributes	No	No	No	-
Assay	Yes	Yes	Yes	Affects the therapeutic effectiveness
pH of product	Yes	Yes	Yes	Affects the stability of the drug and is essential for parenteral administration
Particle size	Yes	Yes	Yes	Affects drug release rate, plasma circulation, and clearance rate
Zeta potential	Yes	Yes	Yes	Affects formulation stability, plasma circulation, protein binding, and uptake by organs
% Entrapment efficiency	Yes	Yes	Yes	Affects therapeutic effect
% Drug loading	Yes	Yes	Yes	Affects release rate and patient compliance
Drug release	Yes	Yes	Yes	Affects therapeutic effect and pharmacokinetic parameters
Pharmacokinetics	No	No	No	It is dependent on the other quality attributes
Stability	Yes	Yes	Yes	Affects therapeutic effect
Storage	No	Yes	No	-

Container closure system	No	Yes	No	-
Administration/ Labelling	No	Yes	No	-

**Does a change in formulation/process parameter affect the quality attribute of a product?*
***Does failure to meet the quality attribute severely affect the efficacy and safety of the product in a patient?*

Various input material attributes and process parameters likely to affect the particular CQAs at each formulation step were identified and the process flow map was created as described in **Figures 1** and **2**. The process flow map gave an overview of the entire formulation process and the factors/causes likely to create variability in the quality attributes of the product. This may assist in finding the probable problem (s) and its solution at each step of the formulation process.

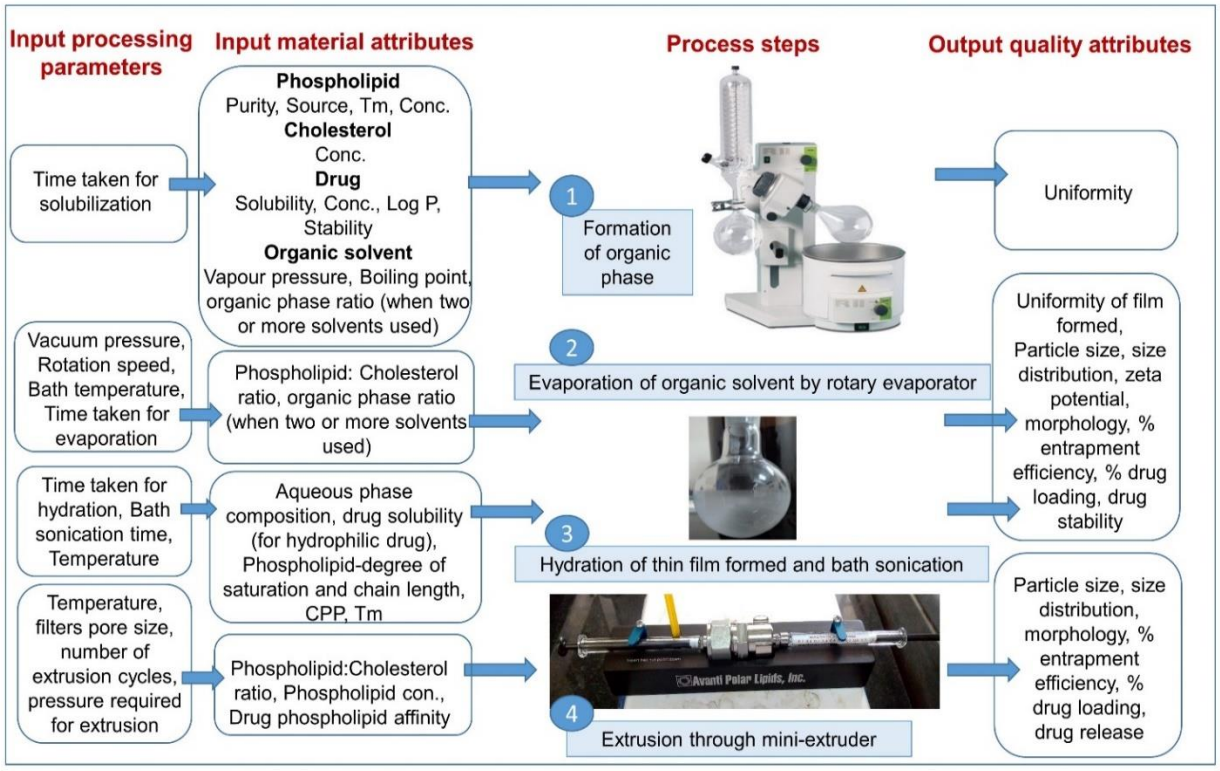

Figure 1: Process flow map for TMZ loaded liposomes using membrane extrusion technique.

(Conc.: Concentration, CPP: Critical packing parameter, Tm: Phase Transition Temperature)

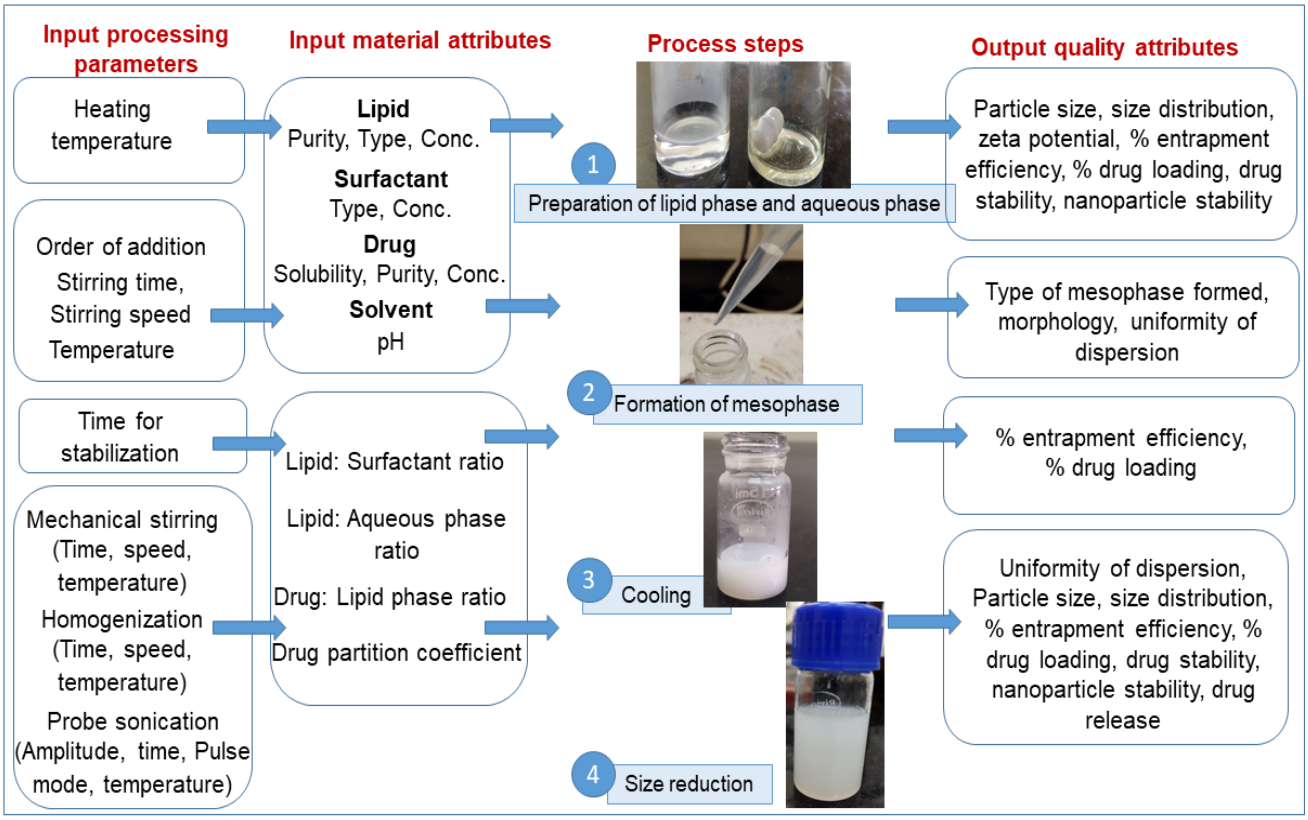

Figure 2: A process flow map for preparation of TMZ loaded LLCs

With the help of relative risk-based matrix analysis and failure mode effect analysis, various critical material attributes and critical process parameters were identified. Thereafter, the effect of various material attributes and process parameters on critical quality attributes was studied in detail. During the formulation of liposomes, the temperature has to be maintained above the phase transition temperature of the phospholipids used. For liposomes, less number of factors were found to be critical, thus the effect was studied individually. For LLCs, the effects were studied with the help of experimental design and Design Expert Software. The data was analyzed using statistics and various model graphs. Design space was also established. The entrapment efficiency of TMZ in these nanocarriers was found to be mainly dependent on the internal to external volume ratio. The optimized formulations were further PEGylated using DSPE-PEG 2000. All the nanocarriers were in the 80-150 nm particle size range with narrow size distribution. The PEGylated nanocarriers carried a negative surface charge as compared to the uncoated nanocarriers **(Figure 3 and 4).**

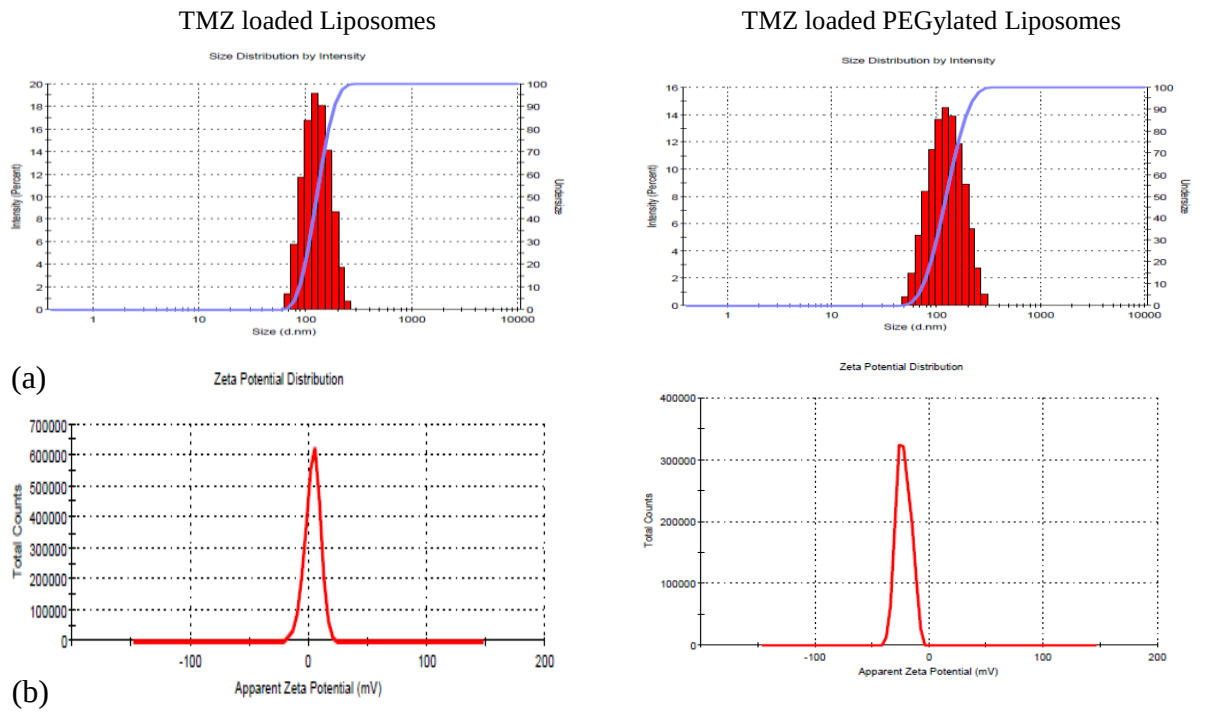

Figure 3: (a) Particle size graphs of TMZ loaded Liposomes and TMZ loaded PEGylated Liposomes (b) Zeta potential graphs of TMZ loaded Liposomes and TMZ loaded PEGylated Liposomes.

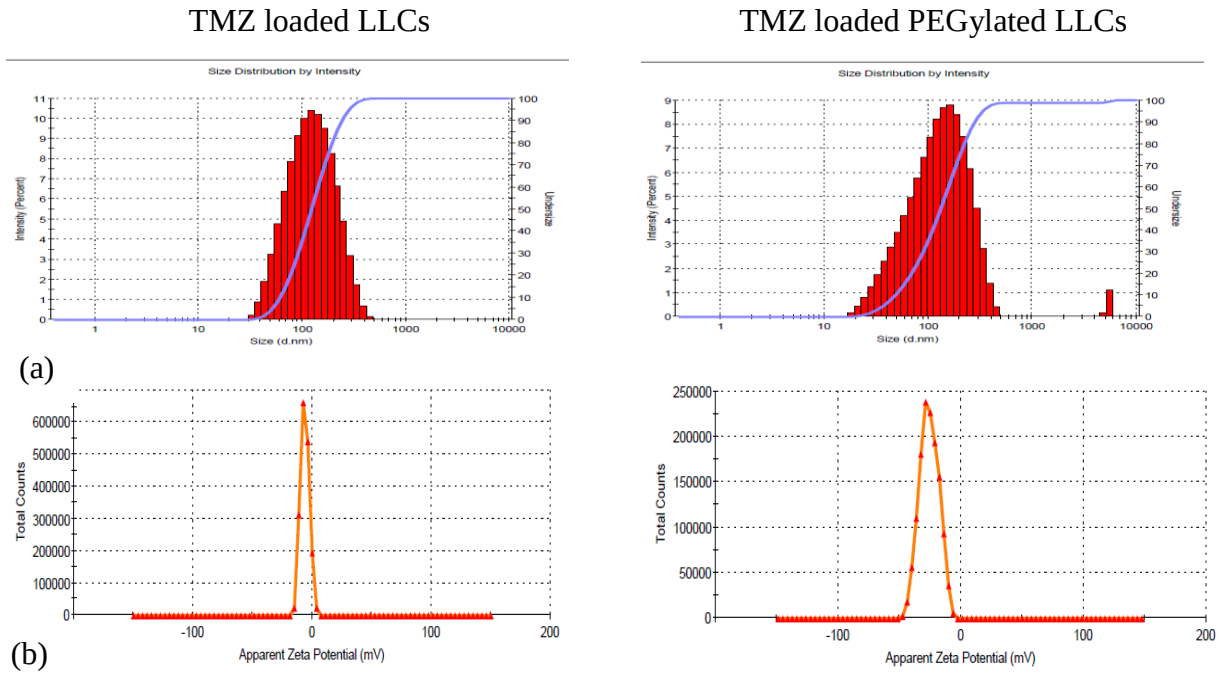

Figure 4: (a) Particle size graphs of TMZ loaded LLCs and TMZ loaded PEGylated LLCs (b) Zeta potential graphs of TMZ loaded LLCs and TMZ loaded PEGylated LLCs.

The average particle size (diameter) obtained with the TMZ-loaded liposomes and PEGylated liposomes was 104.323 ± 10.147 and 110.833 ± 8.062 nm with PDI of 0.141 ± 0.038 and 0.129 ± 0.032 respectively. The average diameter obtained with the TMZ-loaded LLCs and PEGylated LLCs was 100.98 ± 6.14 and 108.15 ± 8.526, with PDI of 0.165 ± 0.05 and 0.283 ± 0.023, respectively. All the nanocarriers were in the 80-120 particle size range with narrow size distribution [11]. The zeta potential of 1.92 ± 2.59 mV and -26.05 ± 4.18 mV was obtained for TMZ-loaded liposomes and PEGylated liposomes, respectively. The near-neutral surface charge of liposomes might be attributed to the neutral phosphatidylcholine groups present. The zeta potential of -4.49 ± 2.19 mV and -20.24 ± 6.42 mV was obtained for TMZ-loaded LLCs and PEGylated LLCs, respectively. The near-neutral surface charge of LLCs can be attributed to the non-ionic GMO molecule present. The PEGylated nanocarriers carried a negative surface charge compared to the uncoated nanocarriers, possibly because of the hydroxyl groups present in the PEG 2000 molecule [12]. The entrapment efficiency obtained with liposomes, PEGylated liposomes, LLCs, and PEGylated LLCs was 45.014 ± 4.835 %, 42.994 ± 3.303 %, 37.46 ± 5.481 % and 40.451 ± 2.561 % respectively [10,11].

The morphology of these nanocarriers was confirmed using various microscopic techniques **(Figure 5).** The fluorescence microscopic image (Figure 2a) confirmed the formation of liposomal vesicles and formation of multilayered vesicles. The SEM image (Figure 2b) confirmed the narrow size distribution and spherical morphology of the formed PEGylated liposomes. The TEM image and the single crystal SAED pattern (Figure 2 c and d) revealed a cubic morphology of the developed PEGylated LLCs [13,14].

The in-vitro drug release studies revealed that TMZ followed first-order kinetics whereas the TMZ loaded formulations were found to follow the Korsmeyer Peppas model. The nanocarriers were found to show a prolonged release up to 24-72 h in comparison to TMZ solution which showed complete dissolution in less than 2 h [15]. The entrapped TMZ has to diffuse through different lipid channels before reaching the outside media. PEGylation did not alter the release kinetics of TMZ from the nanocarriers significantly. When the drug release was conducted in phosphate buffer saline (pH 7.4), initially an increase in TMZ concentration was observed, however, after 2 h, a decrease in TMZ concentration was observed that attributed to the pH-dependent degradation

of TMZ in alkaline pH. In alkaline pH, TMZ undergoes hydrolysis and is converted to 3-methyl-(triazen-1-yl) imidazole-4-carboxamide (MTIC), which in turn converts to 5-amino-imidazole-4-carboxamide (AIC) and a methyl diazonium ion. However, the decrease in TMZ concentration was lower with TMZ-loaded PEGylated liposomes and LLCs as compared to TMZ alone. As TMZ was entrapped inside the liposome and LLCs structure, it was not exposed to the external alkaline pH. Only the released TMZ was exposed and converted to its metabolites. This study indicated that TMZ-loaded PEGylated liposomes and LLCs had the ability to extend the TMZ plasma circulation time when administered intravenously [15,16].

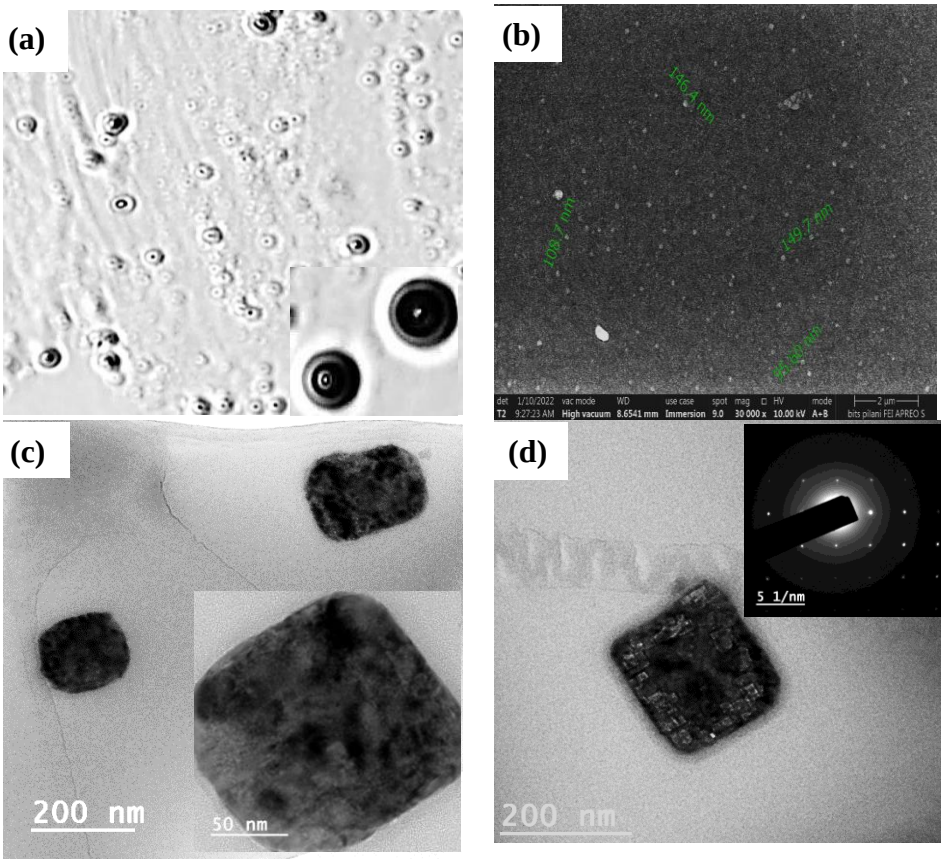

Figure 5: Morphology (a) Fluorescence microscopic image of TMZ loaded PEGylated Liposomes before extrusion (b) SEM image of TMZ loaded PEGylated Liposomes after membrane extrusion (c) TEM image of TMZ loaded PEGylated LLCs (d) TEM image with single crystal SAED pattern of TMZ loaded PEGylated LLCs.

PEGylated LLCs were found to reduce the hemolytic potential of GMO. TMZ-loaded Liposomes and TMZ-loaded PEGylated Liposomes were found to show 1.71 and 1.59 fold more cytotoxicity as compared to TMZ alone. TMZ-loaded LLCs and TMZ-loaded PEGylated LLCs were found to

show 2.22 and 1.54 fold more cytotoxicity as compared to TMZ alone. PEGylated LLCs were found to show more cell uptake in U87 glioblastoma cell lines as compared to LLCs. PEGylated nanocarriers were found to significantly reduce the uptake by macrophage cell lines. PEGylated liposomes and PEGylated LLCs were found to show a 3.47 and 3.18-fold reduced cell uptake in macrophage cell lines compared to uncoated liposomes and uncoated LLCs respectively **(Figure 6)**. When the nanoparticles are administered into the plasma, they are considered foreign particles and taken up by the macrophages through opsonization followed by phagocytosis. It is known that PEGylation of nanoparticles forms a hydrophilic protective layer around the nanoparticles, which prevents opsonization and their uptake by macrophage cells, reducing the clearance rate, and hence providing prolonged circulation of these particles in the plasma [17–19].

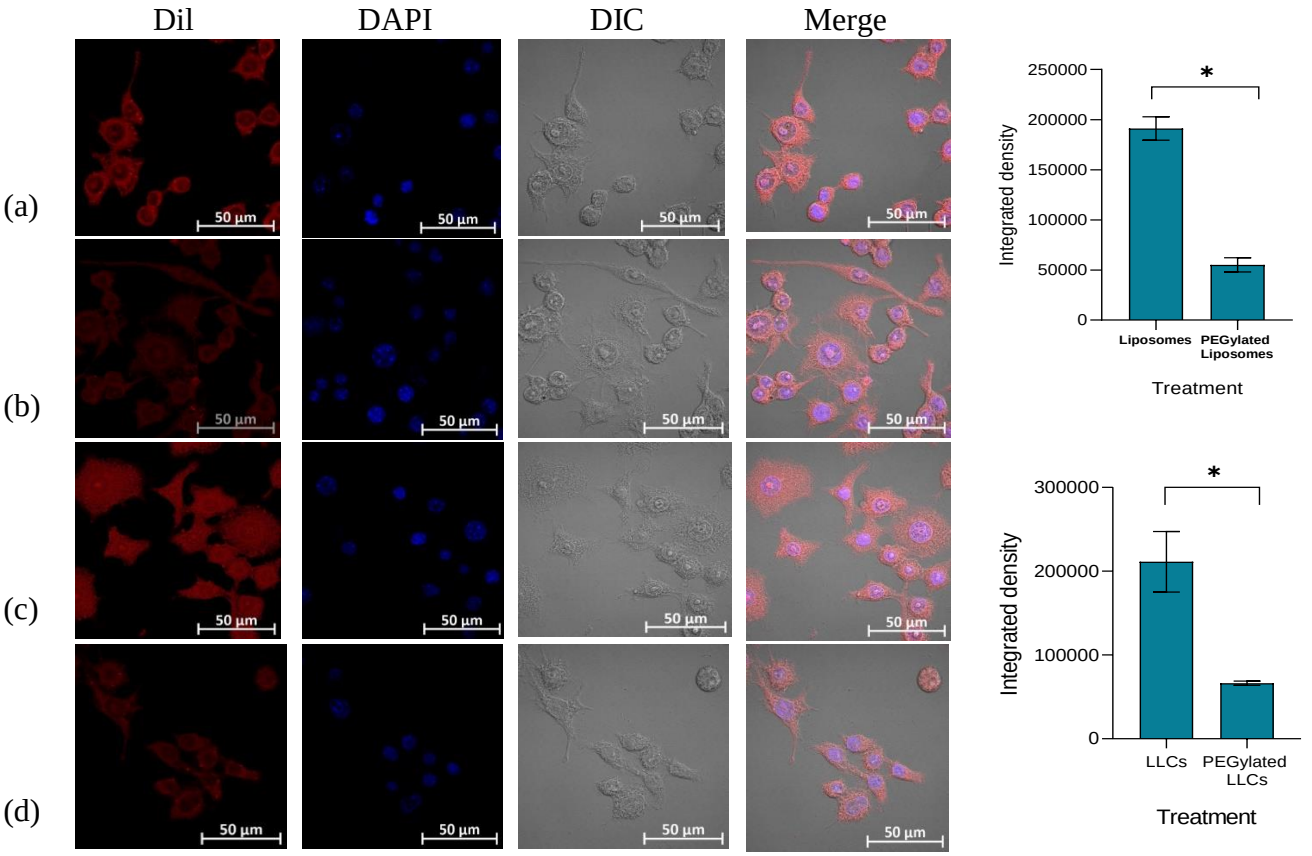

Figure 6: Confocal images obtained for cell uptake studies of (a) Dye loaded liposomes (b) Dye loaded PEGylated liposomes (c) Dye loaded LLCs and (d) Dye loaded PEGylated LLCs in RAW macrophage cell lines after treatment of 4 hours (Data represented as Mean ± SD, n=4) * *statistical significance $p < 0.05$ using t-test*

The pharmacokinetic parameters and biodistribution data confirmed that encapsulation of TMZ within Liposomes and LLCs led to enhanced plasma circulation of TMZ. PEGylated LLCs were able to prolong the plasma circulation time to double whereas PEGylated Liposomes by 1.25 fold in comparison to TMZ solution. The prepared nanocarriers released the encapsulated TMZ in a sustained manner and successfully protected TMZ from plasma pH. This provided delayed conversion of TMZ to the metabolites in comparison to the TMZ alone [20,21]. The pharmacokinetic parameters also confirmed the significance of PEGylation **(Figure 7)**.

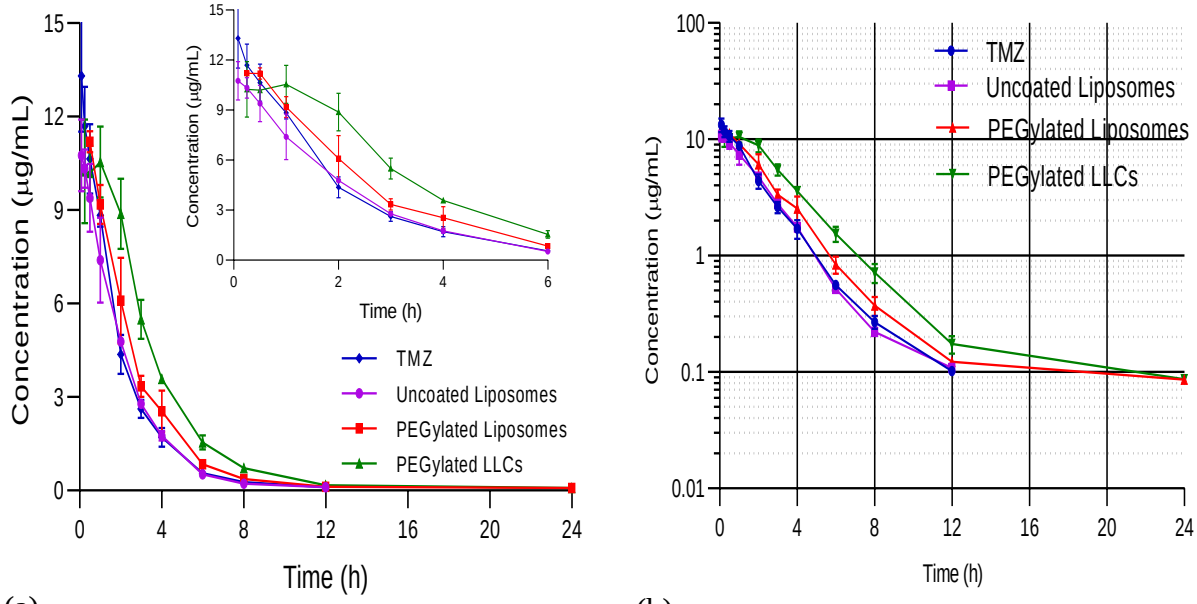

Figure 7: (a) Plasma concentration-time profile and (b) Plasma concentration-time profile plotted on semilog scale obtained for pure TMZ, TMZ-loaded uncoated Liposomes, TMZ-loaded PEGylated Liposomes, and TMZ-loaded PEGylated LLCs following i.v. administration (n =4 expressed as Mean ± SD).

Also, a 1.3 and 1.8-fold reduction in the clearance was observed with PEGylated liposomes and PEGylated LLCs respectively in comparison to the TMZ. Prolonged plasma circulation time along with reduced clearance rate for a drug with short half-life like TMZ is of great significance which was confirmed through biodistribution study. A 2.91 and 6.31-fold increase in brain uptake was achieved at 8 h with PEGylated liposomes and PEGylated LLCs respectively in comparison to the TMZ solution [22,23]. The PEGylated LLCs were found to show better pharmacokinetics and biodistribution in comparison to PEGylated Liposomes in rats following intravenous injection.

Conclusion

The proposed TMZ-loaded PEGylated lipid-based nanocarriers were successfully formulated and investigated. The nanocarriers were prepared using industry-feasible techniques involving a minimum number of steps. PEGylated liposomes and LLCs with particle size ~ 100 nm were prepared for achieving longer plasma circulation. The nanocarriers protected the TMZ from outside pH conditions and provided prolonged drug release. The cell cytotoxicity studies confirmed the cytotoxic potential of these nanocarriers. The cell-uptake studies confirmed that PEGylation significantly reduced the nanocarrier uptake by macrophage cells, thus providing longer plasma circulation time. The pharmacokinetic studies revealed a significant increase in plasma circulation half-life and reduction in clearance of TMZ. Intravenous administration of TMZ-loaded lipid-based nanocarriers successfully resulted in prolonged residence of TMZ in the systemic circulation, with higher residence achieved with PEGylated LLCs followed by PEGylated Liposomes. PEGylation successfully resulted in longer plasma circulation time and enhanced brain bioavailability. The studies confirmed the potential of both nanocarriers in the effective treatment of glioblastoma. Overall, the observations from the pharmacokinetic and biodistribution study indicated that modifying the formulation (either liposomes or LLCs) or changing the phospholipids can modify the distribution of drug to different organs. Thus, selection of specific formulation type may make selective distribution to specific organs.

Future perspectives

- The formulations can be further modified/explored with other novel lipids/ surfactants/ coating agents.
- The optimized formulations can be further investigated in disease animal models.
- The optimized formulations can be further investigated for delivery through other routes like nasal administration.
- The information obtained can be used for the formulation design of other drugs with similar physicochemical properties as of Temozolomide.
- The formulations can be further investigated for clinical application.

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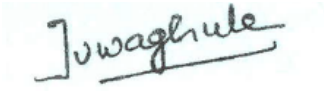
1. **Waghule T**, Swetha KL, Roy A, Saha RN, Singhvi G. Exploring temozolomide encapsulated PEGylated liposomes and lyotropic liquid crystals for effective treatment of glioblastoma: in-vitro, cell line, and pharmacokinetic studies. European Journal of Pharmaceutics and Biopharmaceutics. 2023 May 1;186:18-29. **Impact factor:** 5.589

2. **Waghule T**, Swetha KL, Roy A, Saha RN, Singhvi G. Quality by design assisted optimization of temozolomide loaded PEGylated lyotropic liquid crystals: Investigating various formulation and process variables along with in-vitro characterization. Journal of Molecular Liquids. 2022 Apr 15;352:118724. **Impact factor:** 6.633

3. **Waghule T**, Saha RN, Singhvi G. Exploring microfluidics and membrane extrusion for the formulation of temozolomide-loaded liposomes: investigating the effect of formulation and process variables. Journal of liposome research. 2023 Apr 3;33(2):170-82. **Impact factor:** 5.586

Signature 

Applicant Tejashree Waghule

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