Smart niosomes of temozolomide for enhancement of brain targeting

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Abstract
Drug delivery to the brain is challenging because of the low permeability of blood–brain barrier, and therefore, optimum concentration of chemotherapeutics in the target area specifically for glioblastoma, an aggressive brain tumor, opens a new path of research. To achieve the goal, the oral alkylating agent temozolomide was incorporated into niosomes, and the surface was modified with chlorotoxin, a small 36 amino acid peptide discovered from the venom of scorpion Leiurus quinquestriatus. Active targeting using nanosized particles facilitates an increase in the accumulation of drugs in the cerebri by 3.04-folds. Temozolomide-loaded niosomes were prepared using conventional thin-film hydration method and characterized. Niosomes coated with chlorotoxin were produced with the size of 220 \(\pm 1.45\) nm with an entrapment efficiency of 79.09 \(\pm 1.56\)%.

Keywords
Temozolomide, niosomes, brain targeting, chlorotoxin

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Introduction
Drug delivery to the brain is a major challenge for the formulation scientist because of poor permeability of active pharmaceutical ingredient (API) through the blood–brain barrier (BBB) and active drug efflux transporters of ATP-binding cassette (ABC) which limit the drug targeting of the brain disease management. The ABC transporter P-glycoprotein is highly wide on the cerebral epithelium and plays an important role in this efflux mechanism.

The available treatment strategies for brain tumor-like gliomas are mostly invasive, which involve reversible or irreversible damage to the disruption of the BBB. Localized drug delivery strategy like wafers with stereotactic injection is reported with the danger of infections and entry of foreign matter into the brain. Glioblastoma is a malignant brain tumor with the survival rate mostly less than 2 years. The treatment strategy for brain cancer is usually chemotherapy, but the limitations are the lack of BBB permeability and retention (EPR) and severe side effects on healthy cells. To overcome these problems, a number of approaches have been taken into consideration for the delivery of the drug across the BBB such as the use of prodrugs, carrier-mediated, receptor-mediated, and nanoparticulate drug delivery system.

Niosomes are the bilayer-structured nanoparticles shaped by self-association of nonionic surfactants and cholesterol in the aqueous phase. Niosomes are promising...
candidates for accommodating both hydrophilic and hydrophobic drugs. The surface modification of the niosomes also improves the target specificity for the cancer drug delivery system. According to the literature, the surface-modified niosome formulation with a folate receptor is one of the best candidates for breast cancer targeting. Most of the chemotherapeutic agents exhibit cytotoxic activity, and their presence in the normal cells leads to serious side effects. However, encapsulation of drugs in a suitable vesicular-like niosome can be prefigured to extend the presence of the drug in the systemic circulation, thereby enhancing penetration into the target site with minimum toxicity. The nanocarrier not only prolongs the circulation of the entrapped drug, it also modifies its organ distribution and metabolic stability. From an economic point of view, the development of niosomes involves a simple practical method with minimal quantity of pharmaceutically acceptable solvents.

Temozolomide (TMZ), an oral DNA-alkylating agent, is the first-line choice and the standard care for the treatment of grade IV astrocytoma, glioblastoma, an aggressive brain tumor as single or with radiation. The chemotherapeutic drug leads to double-strand DNA breaks of the cancer cells and causes the cell cycle arrest to initiate apoptosis. In conventional therapy, the drug causes severe bone marrow depressions, inflammation, and birth defects, as well as dose-limiting hematological toxicity in hematopoietic stem cells in patients. In addition, poor solubility and rapid hydrolysis lead to shorter biological half-life, and insufficient biodistribution limits the anticancer activity via the conventional therapy and results in nonspecificity with an increased dose and multiple dosing. Multiple strategies had been taken to improve bioavailability, solubility, and target specificity of the drug, but these strategies did not focus on glioblastoma-specific targeting. This limitation creates a strong need to develop a smart nanocarrier to target specificity of the glioblastoma and thus improving the solubility and stability of TMZ.

To deliver the drug only to the glioblastoma cells and avoid the normal brain tissue damage, the drug-loaded niosomes were modified with the target-specific peptide. Chlorotoxin (CTX), a well-known 36 amino-acid small peptide identified from the venom of the scorpion Leiurus quinquestriatus, has a high affinity for brain, specifically gliomas. The characteristics of the peptide make it a highly potent candidate for targeting the glioblastoma cells of brain tumor. In the current study, niosomes loaded with TMZ and surface-modified with peptide CTX were prepared, and the efficacy was studied in vivo animal model for enhanced targeting gliomas efficiency.

**Materials and methods**

TMZ was obtained as a gift sample from Natco Pharma (Hyderabad, Telangana, India). CTX and cholesterol were purchased from Sigma-Aldrich (Mumbai, Maharashtra, India). U-373 MG glioma cells were provided by the National Centre for Cell Science (NCCS) (Pune, Maharashtra, India). Span, N-hydroxysuccinimidy1 (NHS), and stearyl amine were purchased from S D Fine-Chem Limited (Mumbai, Maharashtra, India). All other chemicals used in the study were of analytical grade.

**Preformation compatibility studies**

**Differential scanning calorimetric analysis.** The compatibilities of the API and other ingredients were examined by differential scanning calorimetric (DSC) analysis (TA Instruments, Bangaluru, Karnataka, India). The samples (3–6 mg) were heated (50–300°C) at a fixed scanning speed (10°C min⁻¹) in sealed aluminum pans under nitrogen atmosphere.

**Preparation of niosomes.** TMZ niosomal formulation was prepared using the modified thin-film hydration method. Briefly, accurately weighed quantities of surfactant and cholesterol in different molar ratios (7:3, 7:4, 7:5, and 7:7) were dissolved in 10 ml of chloroform/methanol mixture (2:1, v/v) in a 250-ml round-bottomed flask to which 1% of charge inducer stearyl amine was added and dissolved. It was allowed to rotate in a flask evaporator at 60–70°C. Further, a vacuum was applied at 437 mbar, and then the flask was further allowed to rotate at 100–120 r min⁻¹ for 1 h to get a thin film. After the removal of the last trace of the organic phase, the layer was hydrated by the addition of 10 ml of phosphate-buffered saline (PBS) containing drug and allowed to rotate at 60°C for 1 h. The resulting niosomal suspension was automatically shaken for 1 h using a shaker at 60 r min⁻¹ at 40°C contributing to the shaping of multilamellar niosomes. The niosomal system was allowed to swell overnight at 4°C. The niosomal formulation was probe-sonicated for 30 min in cycles of 10 min each for three. The un-entrapped drug was got rid of by centrifugation at 13,000 r min⁻¹, and the niosomal deposit was predisposed in millipore water containing 0.5% mannitol as the cryoprotecting agent. The suspension was once again sonication for 5 min and lyophilized to get free-flowing powder.

**Optimization of niosomal formulation**

**Effect of cholesterol.** The surfactant cholesterol ratio is one of the vital factors for entrapment of the anticancer drugs. With the changing ratio of surfactant and cholesterol, the entrapment efficiency of the drug decreases in niosomes. Cholesterol is one of the most common improvers included in the preparation of stable niosomes.

The molar ratio of cholesterol and the surfactant is the first-line choice and the standard care for the treatment of grade IV astrocytoma, glioblastoma, an aggressive brain tumor as single or with radiation. The chemotherapeutic drug leads to double-strand DNA breaks of the cancer cells and causes the cell cycle arrest to initiate apoptosis. In conventional therapy, the drug causes severe bone marrow depressions, inflammation, and birth defects, as well as dose-limiting hematological toxicity in hematopoietic stem cells in patients. In addition, poor solubility and rapid hydrolysis lead to shorter biological half-life, and insufficient biodistribution limits the anticancer activity via the conventional therapy and results in nonspecificity with an increased dose and multiple dosing. Multiple strategies had been taken to improve bioavailability, solubility, and target specificity of the drug, but these strategies did not focus on glioblastoma-specific targeting. This limitation creates a strong need to develop a smart nanocarrier to target specificity of the glioblastoma and thus improving the solubility and stability of TMZ.

To deliver the drug only to the glioblastoma cells and avoid the normal brain tissue damage, the drug-loaded niosomes were modified with the target-specific peptide. Chlorotoxin (CTX), a well-known 36 amino-acid small peptide identified from the venom of the scorpion Leiurus quinquestriatus, has a high affinity for brain, specifically gliomas. The characteristics of the peptide make it a highly potent candidate for targeting the glioblastoma cells of brain tumor. In the current study, niosomes loaded with TMZ and surface-modified with peptide CTX were prepared, and the efficacy was studied in vivo animal model for enhanced targeting gliomas efficiency.
formulation. Increase in the concentration of cholesterol leads to decrease in the size of niosome as well as lower drug entrapment. The increasing concentration of cholesterol creates a competition with the drug for the bilayer formation and reduces the packing space with smaller particle and lower entrapment. Simultaneously, the study also suggests that only in the optimum concentration of the cholesterol the stable bilayer structure was formed with optimum drug entrapment.

**Temperature of hydration.** Hydration temperature of the thin film is another significant factor affecting the entrapment efficiency of a drug in niosomes. In the present study, hydration of niosomes was performed at room temperature (25°C) and at 60°C to look into the effect of hydration temperature on entrapment efficiency, shape, and size. The hydrating temperatures should be maintained above the gel–liquid phase transition temperature. The phase transition temperature of the lipid and the surfactant alters the drug entrapment.

**Effect of sonication time.** Sonication process\textsuperscript{16,20} is an important factor for the achievement of desirable particle size. The main effect of sonication is the cavitation, which is responsible for the particle size reduction and enhancement of the bioavailability.

**Preparation of surface-modified nanoparticles**

The optimized batch was surface-modified with Polyethylene glycol (PEG)-1000 on a magnetic stirrer for 24 h. For the preparation of CTX-conjugated niosomes, the thiol group of CTX was conjugated with the maleimide group of PEG chains on niosomes. The thiolated CTX was incubated with PEGylated niosomal overnight at room temperature. The conjugated niosomal formulations were separated using the Sepharose CL-4B column and re-dispersed in PBS buffer (pH 7.4). The suspension was lyophilized to get the free-flowing powder of CTX-conjugated TMZ niosomes. The conjugation efficiency was measured by bicinechonic acid assay (BCA) protein assay. Briefly, 25 ml of bovine serum albumin (BSA) standards, blank niosome, and conjugated formulation was added in 96-well plates along with the BCA protein assay reagent. After incubation, the absorption was measured via microplate reader at 562 nm. The conjugation efficacy was expressed in percentage.

**Evaluation of niosomal suspension**

**Vesicle size determination and zeta potential.** Vesicle size and zeta potential of TMZ niosome\textsuperscript{16,17} are very important. Alteration of size and charge can prevent the diffusion of the drug to the target site. The size and the charge were measured by a particle size analyzer (Malvern Instruments, Swavesey, Cambridgeshire, United Kingdom) with a zeta potential measurement facility. The polydispersity index (PDI) was used to measure the width of the size distribution.

**Determination of entrapment efficiency.** Encapsulation efficiency (EE\%) for the developed niosomal formulation was assessed using the centrifugation technique.\textsuperscript{21,22} The developed TMZ-CTX-NP formulations (1 ml) were placed in a centrifuging tube (15 ml) and centrifuged up to 15,000 r min\textsuperscript{-1} at 4°C on a Remi cold centrifuge (Remi Equipment Ltd, Mumbai, Maharashtra, India) for 15 min. The diluted supernatant absorbance was measured at 254 nm, and the drug entrapment was calculated using the formula

$$EE\% = \frac{\text{Amount of entrapped drug}}{\text{Total amount of drug}} \times 100$$

**Scanning electron microscopic analysis.** The scanning electron microscopic (SEM) study was done as an evaluation of the external morphology of the developed niosome formulation (JSM-7800F; JEOL, Japan). Samples were diluted with ultrapurified water and spread over a glass sample holder and dried under vacuum. They were subsequently coated with gold and examined under SEM by the refractive index at 30 s runtime.\textsuperscript{20}

**In vitro release studies.** In vitro release of TMZ-CTX-NP from niosomal formulations were studied by the dialysis method. A known amount of niosomes was poured in the dialysis bag (Hi-Media, 12,000–14,000 Da weight cutoff range). In the alkaline stage, the dialysis bag was positioned in a cylindrical beaker containing 100 ml of PBS (pH 7.4). The vessel was placed over a magnetic stirrer (100 r min\textsuperscript{-1}), and the temperature was maintained at 37 ± 0.5°C. Samples were withdrawn at predetermined time intervals and substituted with the fresh medium equilibrated at 37°C, and the test was continued for 24 h. The sink condition was maintained throughout the experiments.\textsuperscript{16,17,22,23} Samples were analyzed using spectrophotometer at 254 nm.

**Release kinetic analysis.** The acquainted data received from the prepared niosomes were analyzed further for fittingness of data in different kinetic models using zero-order, first-order, Higuchi, and Korsmeyer–Peppas models.\textsuperscript{24,25} The correlation coefficient (r), the order of release form, was checked in each case.

**Physical stability studies.** The physical stability of surface-modified niosomes was tested by storing them at refrigerator temperature (4°C) and room temperature (25°C) for 3 months according to the guidelines of ICH.\textsuperscript{26,27} Then the samples were analyzed for change in color, pH, and percentage of drug content at 15, 30, 60, and 90 days after storage.

**In vitro hemolytic toxicity.** Blood samples were collected from rat in evacuated siliconized glass tube containing sodium
citrate. Centrifugation was performed at 1500 r min\(^{-1}\) for 10 min to separate the red blood cells (RBCs); later, cells were washed thrice with saline. The stock solution of the erythrocytes was prepared using saline water such that cell count was 1 \times 10^8 cells ml\(^{-1}\). To obtain 150–1000 \(\mu\)g ml\(^{-1}\) of nanoparticle dispersion and nanoparticles, equal amount of RBC suspension and nanoparticle dispersion were suspended in a microcentrifuge tube. The obtained nanoparticle dispersion and nanoparticles were incubated for 1 h at 37°C. Then, 1% Triton X is used as the positive control, and saline water is used as the negative control. After 1 h of incubation, the tubes were subjected to centrifugation for 10 min at 1500 r min\(^{-1}\). The amount of the released hemoglobin in the supernatant was detected using ultraviolet-visible spectroscopy at 239 nm. The percent hemolysis was calculated by the formula:

\[
\% \text{hemolysis} = \frac{\text{ABS sample} - \text{ABS 0 %}}{\text{ABS 100 %} - \text{ABS 0 %}} \times 100
\]

The absorbance of the supernatant of erythrocyte and scaffold suspension is designated as ABS sample.

ABS 0% is the absorbance of supernatant of erythrocyte and PBS suspension.

ABS 100% is the absorbance of supernatant of erythrocyte and Triton X.

**In vitro cell line study**

U-373 MG glioma cells were used for the in vitro cytotoxicity study. The cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. Cell growth was executed at 37°C in a 5% carbon dioxide (CO\(_2\)) atmosphere.

**Cytotoxicity by SRB assay.** The cytotoxicity of TMZ, TMZ-NP, and TMZ-CTX-NP against U-373 MG glioma cells was measured by SRB assay. Briefly, glioma cells with a density of 1 \times 10^5 cells per well were seeded in a 96-well plate and left for incubation for 24 h under moist environment of 95% air and 5% CO\(_2\) at 37°C. Then, absorbance was measured using Multiskan FC microplate photometer (Thermo Fisher Scientific, Loughborough, United Kingdom) at the wavelength of 540 nm. The data were graphically analyzed.

**Pharmacokinetic studies in rats**

The tissue distribution of the prepared TMZ-CTX-NP nosomal formulation was compared to a drug in oral suspension formulation in Wistar rats. The study was carried out under the guidelines compiled by Committee for the Purpose of Control and Supervision of Experiments on Animals. The study was sanctioned by the institutional animal ethics committee of JSS College of Pharmacy, Ooty, Tamil Nadu, India. Male mice (200–250 g) were divided into three groups of four animals each to perform the tissue distribution profile. Animals were housed individually in the identical laboratory cage and had free access to standard laboratory feed and water ad libitum. The dose of TMZ per animal was calculated according to the body surface area of the animal. The animals were authenticated 45 min after the injection. Blood was collected by cardiac puncture into tubes containing 10% sodium citrate (30 \(\mu\)l) as anticoagulant. Plasma was separated from other blood components by centrifugation at 3000 r min\(^{-1}\) for 20 min and stored at \(-4^\circ\)C. The brain, heart, lungs, liver, spleen, and kidneys were removed from each animal and washed with PBS. The separated organs were separately homogenized in 4 ml PBS using a tissue homogenizer at 13,000 r min\(^{-1}\) for 30 min. The clear supernatant solution was then separated and kept at 4°C until further analysis. Drug concentration in the organ supernatants and in the plasma was measured by a validated high-performance liquid chromatography (HPLC) method. The mobile phase used for the study was ammonium formate buffer (20 mM) and acetonitrile in a ratio of 90:10 v/v at pH 3.0 with the Hyperclone BDS C18 column as the stationary phase. The flow rate of the system was maintained as 0.9 ml min\(^{-1}\) and detected at 316 nm. For extraction, 200 \(\mu\)l of plasma was agitated with 1 ml of HPLC mobile phase for 1 min and left to stand for 15 min for the organic layer to separate. The organic layer was then removed with a micropipette and allowed to evaporate at 70–80°C. The residue was reconstituted with 1 ml of PBS. The peak area was measured at 316 nm. Calibration curves were constructed by spiking blank rabbit plasma samples with a standard volume of TMZ solution. The drug concentration in the analyzed samples was quantified by peak area. The retention time was 8.4 min (Figure No: 7). A calibration curve was linear over the range of 10–420 ng ml\(^{-1}\). The concentration of the drug in organ and plasma was determined using the standard curve generated from TMZ-spiked plasma. The method was validated to ensure accuracy and repeatability (intra-day, inter-day).

**Statistics and data analysis**

Statistical analysis of tissue distribution studies and pharmacokinetic parameters was performed by one-way analysis of variance using GraphPad Prism software (version 3.0) and expressed as mean ± standard deviation. The level of significance was taken at \(p < 0.05\).

**Results and discussion**

**Compatibility study using DSC**

Figure 1 represents the thermogram of TMZ, cholesterol, Span, and physical mixture. The DSC of TMZ drew out an endothermic peak at 207.76°C near to the reporting melting point which is 207°C. Also, it was detected that the endothermic peak of the mixture excogitated the characterization of TMZ alone. Then it was thought to suggest that
there was no evidence of interaction between TMZ and the used excipients.

**Effect of formulation variables and preparation of niosomal formulation**

Cholesterol acts as a critical ingredient in controlling the properties and behavior of the layered niosomes. Surfactant serves as the core material for bilayer formation, not the carrier alone. So, cholesterol allows for the required strength of the bilayer, but itself is incapable of layer establishment. The study revealed that the vesicle size increases linearly with increasing the cholesterol to surfactant concentration (1:1, 1:0.75, 1:0.50, 1:0.25, 3:2, and 3:5) leading to agglomeration. Establishment of blank niosomal formulation without any precipitation was detected at a cholesterol:surfactant ratio of 3:7, 4:7, 5:7, and 6:7, respectively. Low PDI value is a denotation of more homogenous vesicles. Although the PDIs of surfactant:cholesterol formulations of 3:2, 4:5, 2:3, 5:4, 5:9, and 9:5 were cast out due to larger vesicle size and precipitation of cholesterol, the surfactant:cholesterol ratios of 7:3, 7:4, 7:5, and 7:6 were selected for the determination of entrapment efficiency for TMZ, which is summarized in Table 1. It was observed that vesicles prepared with a molar ratio of 7:4 (surfactant: cholesterol) demonstrated the most efficient entrapment of 79.09 ± 1.56% with the sustained release of 83.23 ± 0.42% after 24 h with the particle size of 222.9 ± 2.06 nm. After the BCA assay, the conjugation of CTX with the TMZ-NP was calculated to be 25%.

**Evaluation of formulated niosomes**

**SEM analysis.** The scanning electron microscopic images revealed the spherical nature of niosomes as shown in Figure 2. Sonicated vesicles remained non-aggregated up to 15 days compared to non-sonicated vesicles. The average size of non-sonicated vesicles was 1030.92 ± 10.2 nm, while the size of sonicated vesicles was 220.0 ± 2.0 nm. It was observed that the effect of sonication on vesicles produced smaller size which was 4.5 times lesser than non-sonicated vesicles.

**PDI and zeta potential.** The PDI of niosomes was found to be 0.15 ± 0.031, with the zeta potential of 3.26 mV (Figure 3) representing good homogeneity and electro-photometric stability. The positive charge arises due to the protonation of basic –NH₂ group in stearyl amine. Particles with zeta potential close to zero are less able to be phagocytosed by macrophage. The quality and density of charge on the surface of the niosomes also molds the extent of

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**Table 1. Effect of the process parameters on the entrapment efficiency and the particle size for the niosomal formulation.**

| Process parameters | %Entrapment efficiency | Particle size (nm) |
|--------------------|------------------------|-------------------|
| **Effect of surfactant** | | |
| Span 20 | 50.19 ± 0.7 | 867.67 ± 0.89 |
| Span 40 | 59.89 ± 0.23 | 445.91 ± 1.67 |
| Span 60 | 73.18 ± 0.45 | 296.23 ± 2.56 |
| Span 80 | 41.56 ± 0.5 | 1145.34 ± 1.41 |
| **Effect of cholesterol** | | |
| Cholesterol | 76 ± 0.19 | |
| **Effect of hydration** | | |
| Cetyl alcohol | 55 ± 0.5 | |
| **Temperature** | | |
| 25°C | 48.21 ± 0.57 | |
| 45°C | 62.45 ± 1.34 | |
| 65°C | 72.2 ± 1.56 | |
| **Effect of sonication** | | |
| Sonicated | 220 ± 1.94 | |
| Non-sonicated | 1030.92 ± 2.08 | |

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**Figure 1.** DSC spectrum of TMZ, cholesterol, Span and physical mixture indicating no interaction. DSC: differential scanning calorimetry; TMZ: temozolomide.

**Figure 2.** SEM analysis of TMZ-CTX-NP formulation. SEM: scanning electron microscopic; TMZ: temozolomide; CTX: chlorotoxin; NP: nanoparticle.
biodistribution as well as the fundamental interaction and consumption of niosomes by target cells.

Drug entrapment efficiency. CTX-TMZ-NP entrapment efficiency was tempted by the chemical attraction of the drug into the niosome substantial, thickness of the niosomal bilayer, solubility of the drug in aqueous phase, and compatibility between drug and niosome excipients. High entrapment efficiency was observed in the formulation of a surfactant:cholesterol molar ratio of 7:4 (Table 2). Drug partitioning will happen more easily in highly placed systems of surfactant and cholesterol. In our investigation, we have used Span 60 as the surfactant due to the following conceptions:

(a) Span 60 has the most prominent phase transition temperature.
(b) The length of the alkyl chain of the surfactant is an essential factor in permeability.
(c) Long-chain surfactant brings out high entrapment efficiency.
(d) The longer alkyl chain determines the hydrophilic-lipophilic balance (HLB) measure of the surfactant that successively influences the drug entrapment efficiency. The lower the HLB value of Span 60 (HLB 4.7), the higher will be the drug entrapment efficiency and stability.

| Ration of cholesterol:surfactant | Amount of cholesterol (mg) | Amount of Span (mg) | %Entrapment efficiency |
|----------------------------------|----------------------------|---------------------|------------------------|
| 3:7                              | 21                         | 49                  | 69.5 ± 1.32            |
| 4:7                              | 28                         | 49                  | 79.09 ± 1.56           |
| 5:7                              | 35                         | 49                  | 75.09 ± 0.5            |
| 6:7                              | 42                         | 49                  | 69.93 ± 1.09           |

TMZ: temozolomide.

In vitro drug release and pharmacokinetics. The in vitro release of TMZ from niosomal formulation is shown in Figure 4, which indicates that the rise in the cholesterol molar ratio from 7:3 to 7:5 gradually lowered the efflux of the drug and conformity of the membrane-stabilizing ability. By further review of the data, we can resolve that Span 60 niosomes present alkyl chain length subordinate release. It is to be noted that the in vitro results are

![Figure 3. PDI of the optimized niosomal formulation. PDI: polydispersity index.](image)

![Table 2. Optimized batch of TMZ niosome.](image)

![Table 3. Regression value for various kinetic models.](image)

![Figure 4. Comparative in vitro release profile of TMZ-niosomal formulations in comparison with that of pure drug in phosphate buffer pH 7.4 (mean ± SD, p < 0.05). TMZ: temozolomide; SD: standard deviation.](image)
consistent with those of entrapment efficiency, with the highest entrapment efficiency (79.09 ± 1.56) exhibiting the lowest release after 24 h (T24 h = 83.23 ± 0.42%).

To elucidate the mode and mechanism of drug release from the niosomes, the in vitro data was fitted into various release kinetic models. These results directed that the prolonged release characteristics of niosomes formulation follow zero-order kinetics. The “n” value from the Korsmeyer–Peppas model for a niosomal system was between 0.8193 and 0.9922, which confirms the non-Fickian type of diffusion as presented in Table 3.

Stability study. Stability study results are reported in Table 4. Niosome was stable at 4 ± 1°C. Substantial changes in the drug content, pH, was observed after 60 and 90 days of storage at 25 ± 2°C. In formulation containing Span 60 and cholesterol in a 7:4 molar ratio, the percentage of TMZ held after a period of 3 months at refrigerated condition (4 ± 1°C) was 78.76 ± 2.7% and at room temperature (25 ± 2°C) was 68.8 ± 3.79%. Also, the results indicate that approximately 90% of TMZ was retained in a niosomal formulation for a period of 60 days. The nisomal

**Table 4.** Entrapment efficiency and the formulation pH during the stability study.

| Study condition | 0 day     | 1 month   | 2 month   | 3 month   |
|-----------------|-----------|-----------|-----------|-----------|
| % Entrapment efficiency | Stability at 4°C | 79.09 ± 0.7 | 79.01 ± 1.2 | 78.89 ± 2.3 | 78.76 ± 2.7 |
| Stability at 25°C | 79.09 ± 0.9 | 76.13 ± 2.0 | 72.43 ± 2.7 | 68.8 ± 3.7 |
| pH              | Stability at 4°C | 7.4         | 7.35       | 7.24       | 7.1         |
| Stability at 25°C | 7.39       | 7.27       | 6.98       | 6.93       |

Figure 5. Result representing the hemocompatibility of (a) negative control, (b) blank formulation, (c) pure TMZ solution, and (d) TMZ-CTX-NP at the concentration of 1000 μg ml⁻¹ with the incubation time of 1 h at 37°C. TMZ: temozolomide; CTX: chlorotoxin; NP: nanoparticle.

Figure 6. In vitro cytotoxicity study by SRB assay of TMZ, TMZ-NP, TMZ-CTX-NP against U-373 MG glioma cell lines. Data are represented as mean ± SD (n = 3, p < 0.05). TMZ: temozolomide; CTX: chlorotoxin; SD: standard deviation; NP: nanoparticle.
formulation itself is more stable than the other lipid-based systems. Simultaneous coating of neutral PEG on the surface of the niosomal formulation prevents the aggregation of the charged particle and increases the stability of the formulation.

**In vitro hemolytic toxicity.** The amount of the free hemoglobin in plasma determined spectrophotometrically represents the hemolysis of cells. Positive control (1% Triton X 100) exhibited percentage hemolysis of 4.147 ± 0.65%, whereas negative control (saline water) exhibited percentage hemolysis of 0.023 ± 0.001%, respectively. Whereas in the case of the pure drug, the hemolysis was 0.027 ± 1.75% and for the cross-linked TMZ-CTX-NP 0.019 ± 2.69% when compared with the blank niosomal formulation (0.015 ± 2.38%) at the same concentration of 1000 μg ml⁻¹. The macroscopic image of the hemolytic study is shown in Figure 5. Thus, the result clearly indicates that the formulation behaved well within the permitted limit under the highest concentration exhibited negligible hemolytic potential even at the highest concentration and appeared to be nontoxic to RBCs, validating it as hemocompatible and indicating its safety as drug delivery system.

**In vitro cytotoxicity studies**

The *in vitro* cytotoxic activity (Figure 6) of TMZ, TMZ-niosome, and TMZ-CTX-niosome was evaluated by the SRB assay. Blank formulation exhibited no toxicity to cancerous cells which confirms the safety of the nanoparticles as well as shows that the ingredients use do not contribute to cytotoxicity for the cancer cell line. The pure drug and the conjugated formulation showing the cytotoxicity effect on the cancer cell line indicate that after surface modification of the TMZ-loaded niosome did not
alter its therapeutic efficacy. It is evident that TMZ, TMZ-niosome, and TMZ-CTX-niosome exhibited dose-dependent cytotoxic action.

**In vivo pharmacokinetic studies**

The tissue distribution nature of niosomal formulation showed 3.04-fold increase in TMZ in the brain and 1.97-fold and 1.55-fold decrease in liver and kidney, respectively, compared to the pure drug solution, as shown in Figure 8. After the sixth hour of administration of TMZ-CTX-NP and in suspension form, TMZ concentration in the brain was found to be 217.5 ng as compared to 71.35 ng in the form drug in suspension. The higher concentration of TMZ in the brain will help to achieve an optimal therapeutic effect with smaller doses. Moreover, the hepatic and renal toxicities of the drug will also be reduced with target specific of CTX. From these results, we assume that the increase in brain targeting attributed to the intact TMZ niosomes crossing the BBB by either endocytosis or phagocytosis uptake of a brain. The drug could then be delivered by passive diffusion from endothelial cells to brain cells to generate therapeutic effects. Similarly, the TMZ niosomes exhibited 31.90 and 28.5 ng in liver and kidney as compared to free drugs in suspension form of 62.88 and 48.34 ng. The TMZ-CTX-NP accumulated in the liver and kidney was found to be lower as compared to the drug in suspension form. The average plasma drug concentration–time curves after a single oral dose of TMZ (272.388 mg kg\(^{-1}\)) as suspension and niosomal formulation are shown in Figure 9. The pharmacokinetic parameters of CTX-TMZ niosomal formulation were calculated from the individual curves, and the mean values are presented in Table 5. The niosomal drug dispersion showed significantly (\(p < 0.05\)) higher values for \(C_{\text{max}}\), \(T_{\text{max}},\) AUC and MRT are significantly lower value of elimination rate constant as compared with a free drug in suspension. The increase in the mean residence time (MRT) and area under curve (AUC) values and decrease in the elimination rate constant (Kel) value may reflect the sustained release effect of a niosomal formulation, which correlate with in vitro release study. The significant increase of peak concentration \((C_{\text{max}})\) values may be because of enhanced absorption of the free drug.\(^{35-37}\)

**Conclusion**

The current research focused on the enhancement of the stability of the drug TMZ by incorporating it into the biocompatible, economical formulation. The niosomal formulation strategy not only enhances its stability and sustains

![Figure 8. Organ distribution of the CTX-TMZ representing the highest affinity for the brain indicates that the CTX ligan-coated positively charged TMZ niosome shows better brain targeting capability. TMZ: temozolomide; CTX: chlorotoxin.](image)

![Figure 9. Concentration–time profile after oral administration of CTX-TMZ niosome and TMZ pure drug solution in the rat. TMZ: temozolomide; CTX: chlorotoxin.](image)

| Time (h) | Units | Concentration (ng ml\(^{-1}\)) |
|---------|-------|-------------------------------|
| Pure TMZ suspension | | |
| | | 272.38 ± 0.99\(^b\) |
| | | 300.97 ± 0.95 |
| | | 0.1607 ± 0.01 |
| | | 0.48 ± 0.01 |
| | | 2180.86 ± 0.67 |
| | | 1331.5 ± 0.54 |
| | | 8.464929 ± 0.98\(^b\) |
| | | 3.25 ± 0.78 |

| CTX-TMZ niosome | Pure TMZ suspension | Concentration (ng ml\(^{-1}\)) |
|-----------------|--------------------|-------------------------------|
| | | 217.5 ng |
| | | 18460.88 ± 0.44 \(^b\) |
| | | 4337.9 ± 0.2 |
| | | 8.464929 ± 0.98 \(^b\) |
| | | 3.25 ± 0.78 |

TMZ: temozolomide; SD: standard deviation; \(C_{\text{max}}\): peak concentration; \(T_{\text{max}}\): time to reach peak concentration; AUC: area under the curve; Kel: elimination rate constant; \(t_{1/2}\): half life; AUMC: area under the first moment curve; MRT: mean residence time.

\(a\)Data are represented as mean ± SD.

\(b\)Data are represented as mean ± SD.

\(p < 0.05.\)
release, it’s also one of the unique formulations containing the charge inducer which has an electropositive characteristic to cross the BBB. Surface-bound CTX ligands with the NHS reaction produce a stable and showed higher uptake in glioblastoma cells which are more effective to kill the cancerous cell only in a target-specific manner by enhancing the drug crossing through BBB and releasing the TMZ in the brain in a controlled manner without any significant side effects. The target specificity of the CTX molecule is the target specific for the glioblastoma cells of the brain, releases the drug only in the specific site of action, and is helpful to overcome the conventional drawback of the niosome of non-target specificity. Niosome is the protective carrier to enhance the solubility of the TMZ as well as its smaller size and the lipid nature enhance the drug permeability through the brain. The animal study clearly indicates that the drug-loaded surface-modified niosomal formulation is highly target specific and increases the drug accumulation in the brain without any alteration in the therapeutic characteristics of the drug. TMZ-CTX niosomal formulation gives a new hope for the glioblastoma-affected patients to deliver effective doses of TMZ specifically to GBM cells while minimizing dose-limiting toxicity on other healthy tissues.

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