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Formulation and *In Vitro* Evaluation of PLGA Nanoparticles of Temozolomide

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ABSTRACT

In this study, we formulated and investigated the effects of Temozolomide (TM)/Poly (lactide-*co*-glycolide) (PLGA) nanoparticles on the behaviour of C6 glioma cells. The nanoparticles were fabricated by the emulsifying solvent evaporation, and they were characterized by using X-Ray diffraction, scanning electron microscopy (SEM), transmission electron microscopy (TEM). Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) showed that such nanoparticles had a smooth surface and a spherical geometry. Powder X-ray diffraction (XRD) results indicated that TM trapped in the nanoparticles existed in an amorphous or disordered-crystalline status in the polymer matrix. The release profiles of Temozolomide from nanoparticles resulted in biphasic patterns. After an initial burst, a continuous drug release was observed for up to 1 month. Finally, a cytotoxicity test was performed using Glioma C6 cancer cells to investigate the cytotoxicity of Temozolomide delivered from PLGA nanoparticles. It has been found that the cytotoxicity of Temozolomide to Glioma C6 cancer cells is enhanced when TM is delivered from PLGA polymeric carrier and while Temozolomide powder shows activity only up to 12 hours, where as Temozolomide loaded PLGA nanoparticles shows cytotoxicity in much more enhanced way.

Keywords: Temozolomide (TM); Poly(d,l-lactide-*co*-glycolide) (PLGA); Emulsifying-solvent evaporation; Nanoparticles; Cytotoxicity.

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INTRODUCTION

Alkylating agents play an important role in the treatment of intracalvarium spongioblastoma and malignant tumors of the nervous system in human. Temozolomide (TM) is one of the most effective antineoplastic agents for malignant glial tumor, as its partial ability to cross the BBB, but unfortunately, the use of Temozolomide in clinics is restricted because of its short half-life time (about 1.8 Hr), and with prolong administration it shows an unusual cardiomyopathy, which is caused by the accumulation of this drug. In addition, temozolomide also produces acute toxicity such as bone marrow depression, along with some side effects like nausea, vomiting, fatigue and headache. To avoid treatment-limiting side effects and to get better efficacy, modern therapy requires that the drug reaches the site of action in the most efficient and controlled way, which can be achieved, by using colloidal drug carriers as delivery system such as microemulsions, liposomes, niosomes and polymeric nanoparticles. Considering above issues of conventional formulation, there is need to have a formulation which can be passively targeted by coupling the Temozolomide to a carrier that passively reaches the target organ through the enhanced permeation and retention (EPR) effect, so that to reduce frequent dosing by prolong releasing the drug from well design controlled formulation[1, 2]. In this study, we applied a well-known biodegradable polymers Poly (d,l-lactide-co-glycolide) (PLGA), material, which has history of safe use in pharmaceutical and approved in medical applications by FDA[3]. This biodegradable polymers consider as safe polymer to human body as because it undergoes hydrolysis in the body to produce the original monomers, lactic acid and glycolic acid. These two monomers under normal physiological conditions are by-products of various metabolic pathways in the body. Since the body effectively deals with the two monomers, there is minimal systemic toxicity associated with using these polymer for drug delivery or biomaterial applications[4]. To have stable nanoparticulate formulation, two surfactant are selected in formulation viz. Vitamin E D-α- tocopherol polyethylene Glycol 1000 succinate (TPGS) and Poloxamer 188. TPGS not only act emulsifying agent, but also act as a P-glycoprotein efflux inhibitor, so as an excipient for overcoming multidrug resistance and for increasing the oral bioavailability of many anti-cancer drugs. Poloxamers has ability to incorporate into membranes followed by subsequent translocation into cells, thereby affecting various cellular functions, such as mitochondrial respiration, ATP synthesis, activity of drug efflux transporters, apoptotic signal transduction and gene expression. As a result, poloxamers cause drastic sensitization of MDR tumors to various anticancer agents, enhance drug transport across the blood–brain and intestinal barriers, and cause transcriptional activation of gene

expression both *in vitro* and *in vivo*. The objective of present study is to formulate Temozolomide loaded nanoparticles of with biodegradable polymer PLGA. After formulating the formulation (Temozolomide loaded nanoparticles of PLGA) were exhaustively characterized to understand formulation behavior.

MATERIALS AND METHOD

Poly (lactide-co-glycolide) (PLGA) was purchased from Evonik Bangalore, Temozolomide (TM) received as gift sample from Dr. Reddy's Labs (Hyderabad). Vitamin E D-α- tocopherol polyethylene glycol 1000 succinate (TPGS) and Poloxamer 188 were purchased from Sigma Chemical Bangalore, Rat C6 glioma cell line was obtained from the NCCS, Pune. Other chemicals and solvents used in this study were of analytical grade.

Formulation of PLGA nanoparticles of Temozolomide[5, 6, 7]

PLGA nanoparticles of Temozolomide were prepared by dissolving Temozolomide (50 mg) and PLGA (100 mg) in organic solvent methylene chloride (10 mL). This obtained mixture was emulsified by high speed shear homogenizer (Ika Lab. Instruments, Italy) under an agitation of 6000 rpm for 5 min, to 80 ml of aqueous phase containing 0.1% (w/v) Vitamin E d tocopherol polyethylene glycol 1000 succinate (TPGS) and 0.05% (w/v) Poloxamer 188 (saturated with TM beforehand). The obtained primary emulsion was then passed through a high pressure homogenizer (GEA Niro Soavi Panda Plus 2K) at 1000 bar for 20 min. The final product was collected by filtration and vacuum dried. The formulation is optimized with different concentration of Drug and polymer along with different concentration of both the surfactant i.e. TPGS and Poloxamer 188[8]. Process Optimization done with considering to main parameter which was found to be milling time & milling pressure[9, 10].

Characterization of nanoparticles

FT-IR spectroscopy

To understand compatibility between drug and polymer, FT-IR spectrum of Temozolomide PLGA and physical mixture along with final formulation was recorded using FT-IR Spectrophotometer (Shimdzu, Japan) between the ranges of 600-4000 cm^{-1}. It was then compared with the FT-IR-reference spectra of Temozolomide**.**

Particle size distribution

Particle size distribution of the Nanoparticles was determined by Malvern Mastersizer 2000 laser diffractometer (Hydro 2000, Malvern Instruments Ltd., Worcestershire, UK) using a wet sampling system. To analyze the sample, nanoparticles taken in 0.5 mL in 50 mL centrifugal tube, where 20

mL of water added. This mixture is mixed well by cyclo mixer for 1 min. Refractive index of 1.5 and an absorption index of 1.00 was placed. Before placing the sample ensured that equipment is properly cleaned. Sampling start by filling the sample holder with milli 'Q' water, to this sample was added by dropper till the obscuration reach to 7-8%. Sample was analyzed in three measurements with average reporting.

Particle Surface Morphology

Surface morphology of Temozolomide nanoparticles were observed by scanning electron microscopy (SEM, model S-2250N, Hitachi, Japan) and transmission electron microscope TEM (JEM-2100HC;JEOL, Tokyo, Japan) in order to examine the morphology and size of the nanoparticles.

Entrapment Efficiency

The entrapment efficiency is defined as the percentage of the actual mass of drug encapsulated in the polymeric carrier relative to the initial amount of drug loaded. In the determination of the entrapment efficiency, nanoparticles were accurately weighed and dissolved in a certain volume of acetonitrile via sonication till complete solubility. The drug content of each sample was then analyzed using UV spectrophotometer at λmax 327 nm. The entrapment efficiency (EE) was calculated from the following equation

EE (%) = Dm × 100/ Dt

Where Dt is the amount of Temozolomide used for the preparation and Dm is the amount of Temozolomide in the vacuum dried nanoparticles.

Percentage Yield

The percentage yield of different formulations was determined by weighing the nanoparticles after drying. The percentage yield was calculated as follows

% yield = Total weight of Nanoparticles/Total weight of Drug and polymer* 100

Each determination was made in triplicate.

X-ray Diffraction

The crystalline form of the drug dispersed in the nanoparticles and in the physical mixture of drug-polymer was analyzed by X-ray powder diffractometry (D/MAX-III B, Rigaku, Japan). Diffraction patterns were obtained by using X-ray diffractometer with a radius of 240 mm. A system of diverging and receiving slits of 1° and 0.1mm respectively was used. The pattern was collected with 40 Kv of tube voltage and 30 mA of tube and scanned over the range of 5-60.

***In-vitro* Release Studies**

The *in vitro* release of Temozolomide loaded nanoparticles was measured in phosphate buffer (PBS, pH 6.8) in triplicate at temperature of 37°C. The *in vitro* release was studied using a dialysis bag diffusion technique. The Temozolomide loaded nanoparticle was placed in cellulose dialysis bags with a 12,000 molecular weigh cut off (Sigma) which were sealed at both ends, and then the bags were dipped into the crystallizing dishes containing 200 mL phosphate-buffered saline (pH 6.8), which were maintained at 37°C and constantly stirred at 100 rpm. The crystallizing dishes were closed to prevent evaporation of the release medium. At regular time intervals, 2 mL of dissolution medium was withdrawn and the same volume of fresh phosphate buffered saline was added accordingly. The amount of Temozolomide in the medium was measured by UV spectrophotometer analysis. The release study was carried out for 30 days, and sampling done at interval of 0, 1, 2, 4, 6, 10, 12, 14, 16, 20, 24, 28, 30 days.

Zeta potential

Surface charge of formulated PLGA nanoparticle were measured on a Nano-ZS Zetasizer dynamic light scattering detector (Malvern Instruments, Malvern, UK) equipped with a 4.0 mW internal laser. All measurements were performed at 25°C at a scattering angle of 173°. Prior to measurement, the formulation were diluted with deionized water to an appropriate concentration for better measurement. Values of the particle sizes and zeta potentials are presented as mean ± standard deviation (SD) from three replicate samples.

Cytotoxicity Study

Cytotoxicity of polymeric nanoparticles of Temozolomide drug delivery system along with Temozolomide solution was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on C6 glioma cell line to predict the possible dose response of Temozolomide-loaded PLGA nanoparticles. The cytotoxicity assay was conducted by using MTT assay, which is commonly used to quantify the living cells which are still metabolizing. The cell viability was determined by a microplate reader. Cells were transferred to 96-well plate first to ensure 1×10^4 cells per well. Medium was changed every other day until 80% confluence was reached. Then the medium was changed with 100 µL medium with Temozolomide loaded PLGA nanoparticles. The plate was incubated for 1, 2 and 3 days. One row of 96-well plates was used as control without adding nanoparticles. At different intervals, suspension was removed and the wells were washed thrice using PBS. Ten microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and 90µl medium were then added to the wells. After incubation for around 3-4 h, solution was removed, leaving the precipitate. One hundred

microliters of DMSO was then added to the wells before the plate was observed using microplate reader. Cell viability was determined by the following equation:

Cell viability (%) = Abs test cells/ Abs control cells × 100

where Abs test cells and Abs control cells represent the amount of formazan determined for cells treated with the different formulations and for control cells (non-treated), respectively. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

FT-IR spectroscopy

FT-IR spectroscopy of the drug, Temozolomide present in the formulation were confirmed by FT-IR spectra. The characteristics peaks due to pure Temozolomide have appeared in nanoparticle spectra, without any remarkable change in their position after successful formulation by solvent evaporation, indicating no chemical interaction between Temozolomide and used polymer PLGA.. It also confirmed the stability of drug during solvent evaporation process (Figure 1).

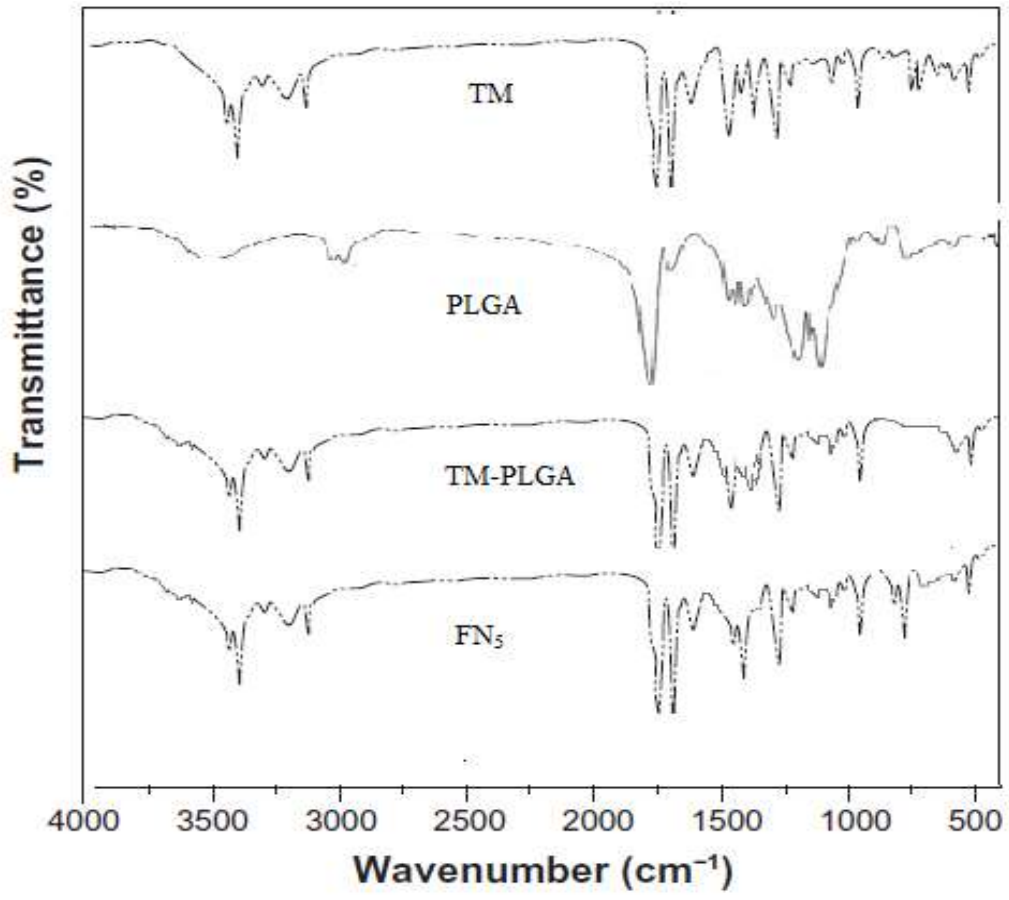

Figure 1: FT-IR Spectrum: Pure Temozolomide (TM), PLGA, Physical Mixture and Nanoparticulate Formulation

Particle size distribution

In formulation of PLGA nanoparticle of Temozolomide it is found that, PLGA nanoparticle shown particle size distribution 256nm to 683nm. The mean particle size of nanoparticle was increased with increasing the milling time and applied milling pressure. It is also found that if polymer concentration not enough in formulation then it also lead to precipitation of drug from formulation which is responsible for higher particle size distribution. Finally the process parameter indicated that milling time and milling pressure play major role in getting smaller size nanoparticles.

Particle Surface Morphology by scanning electron microscopy and transmission electron microscope

Nanoparticles were spherical with no visible major surface irregularity. Few wrinkles and inward dents were appeared at the surface. It may be due to the collapse of the nanoparticle during the *in situ* drying process but they showed good ability on the surface of the medium indicating intact surface. While nanoparticle formed entirely of PLGA were observed to be fairly spherical with nearly regular surface. The SEM and TEM photographs revealed the absence of crystals of the drugs on the surface of the hollow nanoparticle, indicating uniform distribution of the drugs in the walls of the hollow nanoparticle. Photographs also indicated the presence of minute pores on the surface of the hollow nanoparticle.

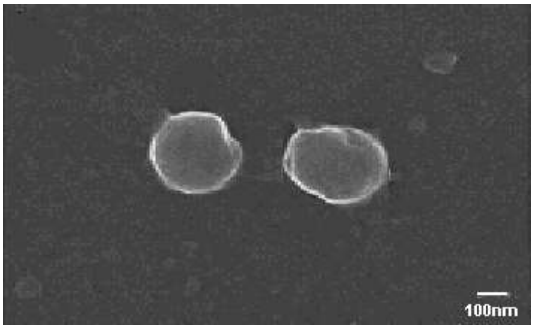

Figure 2: Scanning Electron Microphotograph of Nanoparticulate Formulation at Higher Magnification

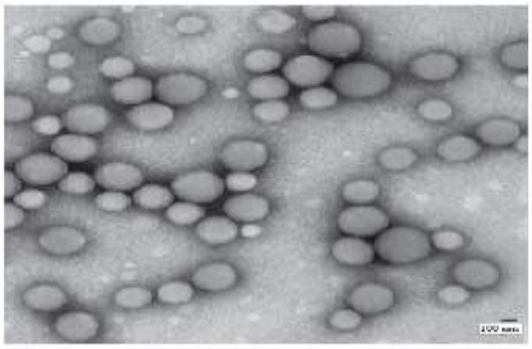

Figure 3: Transmission Electron Microscope of Nanoparticulate Formulation

Entrapment Efficiency

The Entrapment Efficiency of PLGA nanoparticles of Temozolomide range from 71.26 %to 76.45%. It indicates good entrapment efficient of the formulation which found more as there is increase in polymer concentration. Entrapment efficiency also found low in few experiment where formulation is prepared with low concentration of primary surfactant, this is because of if surfactant is not available in enough concentration to reduce interfacial tension of drug molecule hence drug leach out and precipitated resulted in low entrapment of drug in formulation. After optimization i.e. after finalizing the concentration of polymer and surfactant it is found that entrapment efficiency of the PLGA formulation found to be good ensuring that there is no precipitation of drug.

Percentage Yield

The percentage yield of PLGA nanoparticles of Temozolomide ranges from 88.67% to 92.65%, All the formulation of Temozolomide nanoparticle PLGA shown that there is good percentage yield received with all the approach applied in formulation development of polymeric nanoparticle of Temozolomide the reason for this is the formulation with using the High pressure Homogenizer is direct top-down approach hence the input quantity was consistently meeting with output quantity with minor loss which occurred due to sampling and process loss (machine dead volume, tubing loss).

X-ray Diffraction

The X-ray powder diffraction patterns revealed that the intensity of the peaks for the pure drug was sharp. But when it was incorporated into the nanoparticle, the drug peaks showed a loss of sharpness due probably decreased crystallinity of the drug. The X-ray patterns (measured by D/MAX-III B, Rigaku, Japan) of nanoparticle showed only the characteristic peaks of polymer, but no drug peaks. Thus, the drug is not in a crystalline form. These observations are in good agreement with the SEM pictures (no drug crystals visible). Thus, it can be concluded that the drug is not present in a crystalline form in the nanoparticle. It is partly dissolved within the polymer and partly in the amorphous form distributed throughout the system.

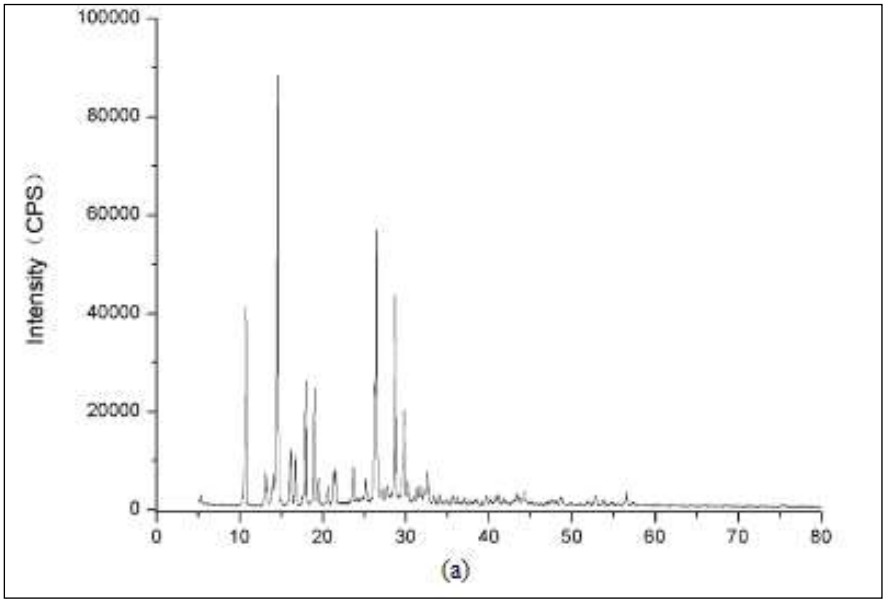

Figure 4: X-ray patterns of (a) pure Temozolomide

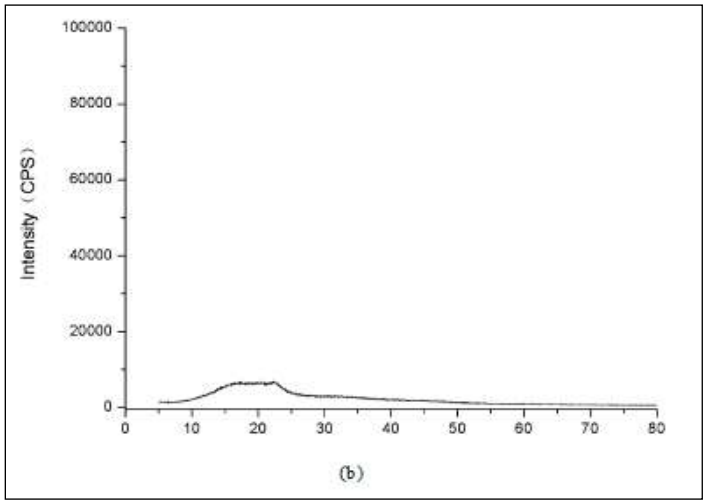

Figure 5: X-ray patterns of (b) pure PLGA materials

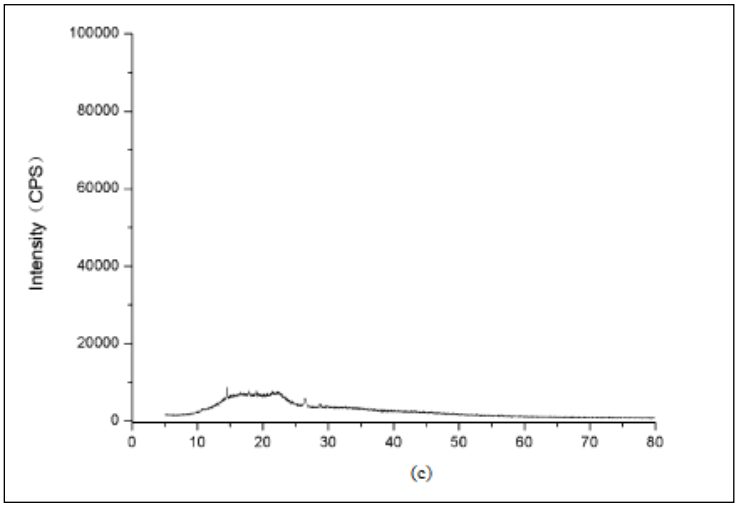

Figure 6: X-ray patterns of TM loaded PLGA nanoparticles

Zeta potential

A physically stable Nano-suspension solely stabilized by electrostatic repulsion will have a minimum zeta potential of ±30 mV. To evaluate the surface charge of polymeric nanoparticles of Temozolomide zeta potential is measured (Zeta potential was measured by Nano-ZS Zetasizer Malvern Instruments, Malvern, UK) and it is found to be value of -33mV for PLGA nanoparticle of Temozolomide.

***In-vitro* Release Studies**[6, 11]

The release profiles of TM from TM-loaded PLGA nanoparticles particles are shown in Figure 7. The release rate of TM from PLGA nanoparticles increased with the increase of TM loading amount and the period of 100% Temozolomide got released was up to 30 days. It showed an initial burst in the first day. Initial burst also increased with the increase of TM loading amount. The reason of initial burst could be due to diffusional release of drug particles on the surface of nanoparticles and a higher drug-loading amount resulted in a high amount drug particle on the surface of nanoparticles. The release rate and pattern of drug from polymer matrix is mainly dependent not only on diffusion of drug through the matrix but also on the degradation of biodegradable polymer. Therefore, drug loading amount, molecular weight, and monomer ratio of copolymer are the major factors affecting the drug release rate and pattern. A period of sustained release estimated from approximately up to 30 days while release amount increased continuously. This release pattern is mainly dependent on the diffusion of the drug through the polymer matrix that has many channels due to the polymer degradation after the water uptake.

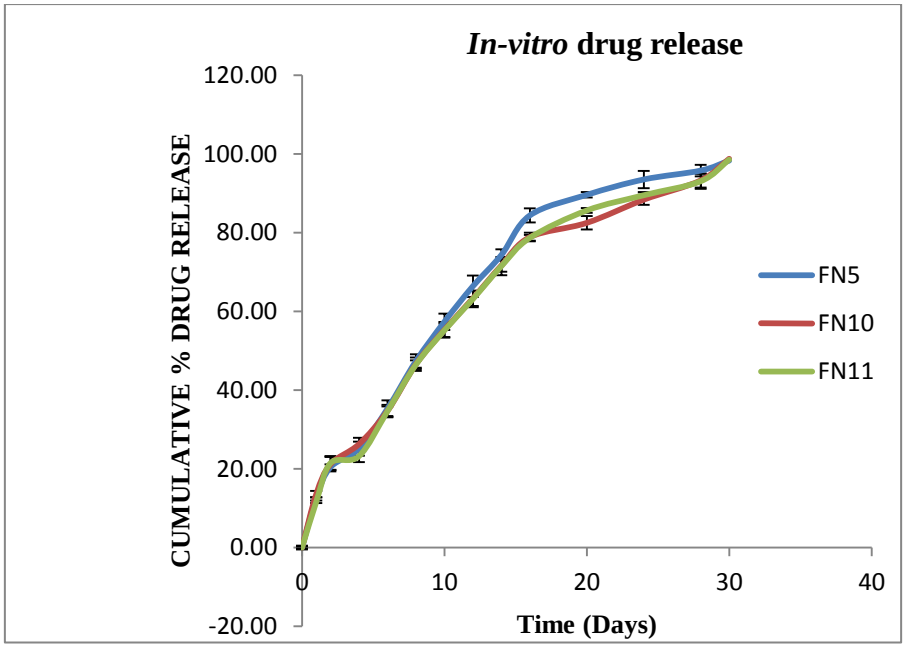

Figure 7: Zero Order Release profile of PLGA nanoparticles of Temozolomide

Cytotoxicity Study[12]

The cyto toxicities of the Temozolomide loaded PLGA nanoparticles were evaluated by assessing cell viability using the MTT assay on glioma C6 cell lines. To facilitate the basis for comparison, cells were incubated with concentrations of nanoparticles that contained the same amount of drug as that of free Temozolomide powder sample with Temozolomide concentration of 20µg/ml. The cyto toxicities of the Temozolomide loaded PLGA nanoparticles and Temozolomide powder are shown in Figure 7. In the case of Temozolomide loaded PLGA nanoparticles, all the samples inhibited the cell growth for all the time course tested. In the case of free of Temozolomide powder, it did not reveal cytotoxicity to the C6 cells after 12 h. This observation shows that the Temozolomide-loaded PLGA nanoparticles have higher cytotoxicity than Temozolomide powder because of Temozolomide powder is found to be effective for very short duration i.e. up to 12 hour hence here repeated dosing is required for maintaining cytotoxicity against glioma C6 cell. It has been found that the cytotoxicity of Temozolomide to Glioma C6 cancer cells is enhanced when TM is delivered from PLGA polymeric carrier, whereas activity of TM powder disappeared within 12 hr.

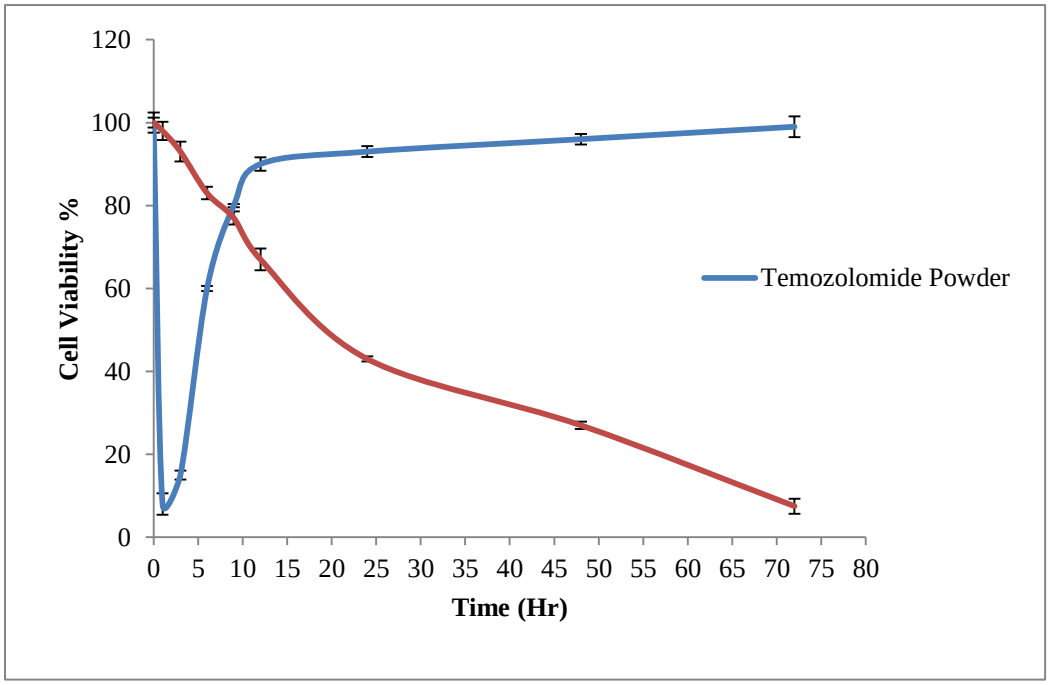

Figure 8: Cytotoxicity of i) PLGA nanoparticles of Temozolomide and ii) Temozolomide Powder

CONCLUSION

Temozolomide loaded PLGA nanoparticles were prepared by emulsifying-solvent evaporation method in reproducible manner. Several preparation parameters, such as initial drug loading,

polymer concentration, and stirring rate played a predominant role in the preparation. Microparticles had spherical shape. From SEM, TEM and X-ray results, it appeared that Temozolomide trapped in the nanoparticles existed in an amorphous or disordered-crystalline status in the polymer matrix. The release profiles of Temozolomide from nanoparticles resulted in biphasic patterns. The release rate of Temozolamide from PLGA nanoparticles increased with the increase of Temozolomide loading amount and the release of Temozolomide was almost finished at 30 days. The results of cytotoxicity test showed that the cytotoxicity of TM to Glioma C6 cells could be enhanced when Temozolomide is delivered from polymeric device. However, despite the results obtained, due to cell culture limitation, long-term reproductive survival of tumor clonogens can only be determined using further *in-vivo* animal tests.

REFERENCES

1. Malcolm J.M., Darkes, Greg L. Plosker and Blair Jarvis, 2002. Temozolomide: A Review of its Use in the Treatment of Malignant Gliomas, Malignant Melanoma and Other Advanced Cancers Am J Cancer; 1 (1): 55-80.
2. Nathalie Wauthoz, Philippe Deleuze, Julien Hecq, Isabelle Roland, *et al. In-vivo* assessment of temozolomide local delivery for lung cancer inhalation therapy Eur J Pharma Sci 2010;39, 402–411.
3. Sarbari Acharya, Sanjeeb K. Sahoo., PLGA nanoparticles containing various anticancer agents and tumour delivery by EPR effect. Advanced Drug Delivery Reviews 2011;63, 170–183
4. Wang Y, Fel D. Controlled release system containing Temozolomide. United state Patent Application Publication 2005 (US 2005/0244494 A1)
5. Soppimath KS. Biodegradable polymeric nanoparticles as drug delivery devices, J. Control. Release 2001; 70, 1-20.
6. Yunni Yu, Songwei Tan et al., Antitumor activity of docetaxel-loaded polymeric nanoparticles fabricated by Shirasu porous glass membrane-emulsification technique, Int J Nanomedicine, 2013:8; 2641–2652.
7. Zhang Z, Lee SH, Gan CW, Feng SS. In vitro and in vivo investigation on PLA-TPGS nanoparticles for controlled and sustained small molecule chemotherapy. Pharm Res. 2008;25(8):1925–1935.
8. Zhang Z, Feng SS. Self-assembled nanoparticles of poly(lactide)–vitamin E TPGS copolymers for oral chemotherapy. Int J Pharm. 2006;324(2):191–198.

9. Rowe RC., Sheskey PJ, Quinn ME. Handbook of Pharmaceutical Excipient, London, Pharmaceutical press 2009.
10. Dongyong Zhang, Ang Tian, Xiangxin Xue, Mei Wang, et al., The Effect of Temozolomide/Poly(lactide-co-glycolide) (PLGA)/Nano Hydroxyapatite Microspheres on Glioma U87 Cells Behavior.; Int. J. Mol. Sci., 2012; 13, 1109-1125;
11. Guihua Huanga, Na Zhang, Xiuli Bi, Mingjin Dou. Solid lipid nanoparticles of temozolomide: Potential reduction of cardial and nephric toxicity. Int J Pharma 2008; 355, 314–320.
12. He Zhang, Shen Gao. Temozolomide/PLGA microparticles and antitumor activity against Glioma C6 cancer cells *in vitro*. Int J Pharma 2007; 329, 122–128.

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