The glutathione-conjugated methylene blue loaded nanoparticles and glutathione-conjugated coumarin loaded nanoparticle were constructed in the present work as biodegradable carrier for brain-specific drug delivery along with evaluation of its *in-vitro* permeation properties. Nanoparticles of PLGA was prepared by reacting it with glutathione using EDAC as a linker, which was also characterize for particle size analysis. The *in-vitro* study performed for permeation property of developed nanoparticles using Trans well co-culture of C6 (rat astrocytoma) cells and RBE4 (rat brain endothelial) cells showed significant permeability of glutathione conjugate nanoparticle. Significant *in-vitro* permeability suggest that glutathione serve as a better vehicle to deliver paclitaxel in brain during brain cancer therapy.

**Keywords:** Glutathione, Paclitaxel, Brain cancer, Nanoparticle
Introduction

Brain cancers, primarily glioblastomas, present with poor prognoses in most patients and have very high clinical malignancies [1]. Central nervous system (CNS) tumors are associated with a number of undesirable side effects, namely nausea and vomiting, headaches and seizures [2]. Gliomas are mainly primary tumors which arise from glial cells; however, there are instances of metastasized breast and lung cancers that induce the formation of brain tumors [2,3]. Gliomas are classified into three main categories by the World Health Organization (WHO): astrocytomas, oligodendrogliomas and oligo-astrocytomas; and each of them is graded from I to IV with an increasing level of malignancy [3]. Although, a number of drugs express promising \textit{in-vitro} efficiency against brain cancers, the therapeutic outcome of these drugs are observed to be limited [4]. This may be due to the Blood Brain Barrier (BBB), which develops through the interactions of endothelial cells, astrocytes and pericytes; resulting into formation of tight junctions in the endothelial cells, which prevents the entry of the drug and other substance into brain. BBB is the major hurdle for variety of anti-cancer drug in the treatment of brain cancer.

Paclitaxel is proven potent anti-cancer drug for brain tumor; however, the permeability through the blood brain barrier is the rate limiting parameter for paclitaxel drug delivery. Paclitaxel is commercially administered as Taxol, belongs to the taxane family of drugs, which is derived from the bark of the pacific yew tree (\textit{Taxus brevifolia}) and has been clinically used against a number of cancers including ovarian and breast cancers and particularly significantly active against malignant gliomas and metastasis [1,2,5,7]. Paclitaxel has a unique mechanism of action that it promotes the assembly of microtubule which in turn prevents depolarization for cell division in the G2 or M phase of cell mitosis causing the cell death [3]. However, specific structural property of brain anatomy and limited permeability of paclitaxel reduces the access of drug at brain tumor. Additionally, Paclitaxel structure is also the subtract for multidrug resistant protein, which express p-glycoprotein (p-gp). Such expressed proteins like p-gp transporter efflux-out the paclitaxel with their transport mechanism [7, 8]. Hence, in conclusion, variety of research it has been proved that permeability of paclitaxel in brine is limiting parameter for brain cancer therapy. According to Fellner et al [6] 90 \% of brain tumor was observed to be reduced by administration of paclitaxel with p-gp inhibitor as compare to paclitaxel alone.

The poor aqueous solubility of paclitaxel due to macro-molecular structure, limits the development of conventional intravenous injections to improve bioavailability at site of action. To improve the solubility of paclitaxel, the co-solvency approach have been explored, whereby 1:1 volume ratio of Cremophor EL and ethanol has shown improved pharmaceutical
However, cremophor EL has been shown to cause hypersensitivity reaction, which limits the clinical application. Although, paclitaxel has been successfully used in a number of different cancers, its use in brain cancers has been limited due to the blood-brain barrier (BBB), which impedes the permeation of possible toxins into neural tissue. To surmount the BBB’s challenge to brain-targeted drug delivery, many attempted has been like liposomes, nanoparticles, micelles and conjugates etc. [9]. After variety of attempts, few formulation (i.e. Xyotax) has been proved as a promising against in phase-II and phase-III clinical trials for brain metastasis [10]. However, the review from the scientist does not expect its efficiency to deliver paclitaxel in to the brain for brain cancer. Therefore, the in-depth research is still required to deliver paclitaxel in brain with high efficiency. The Aim of this present work is to develop efficient carriers, which are able to deliver anti-cancer drug in to the brain for brain cancer therapy.

In present investigation, glutathione was explored as a coating material for efficient delivery of paclitaxel nanoparticle in to the brain for brain cancer. Out of number of mechanisms proposed to induce the trans-BBB permeation of drugs, a glutathione method has shown success in in-vitro and in-vivo studies [11, 12]. Glutathione is the substrate for p-gp protein which is express in brain and therefore the nanoparticles conjugated with glutathione may be able to cross BBB. Therefore, it was predicted that conjugation of glutathione with nanoparticles loaded with methylene blue as well as nanoparticles loaded with coumarin may improve the permeation of drug through BBB.

**Material and method**

**Material**

PLGA was supplied by CHS pvt. Ltd., Acetone and Poloxamer 188 were purchased from sigma eldritch, EDAC was procured from MP Biomedical. Glutathione, Coumarine, Methylene blue were supplied by Venus Chemicals. Methanol used was of analytical grade. Deionized water used for further experiments was obtained from Millipore.

**Preparation of methylene blue loaded nanoparticles**

The methylene blue loaded PLGA nanoparticles were prepared by nanoprecipetation method proposed by Patel N et al. [13] Initially, the PLGA was dissolved in acetone with pluronic F-108. Methylene blue was was separately mixed with acetone using magnetic stirrer. Organic solvent containing PLGA, pluronic F-108 and methylene blue were added drop wise at the rate of 2ml/min into the deionized water under continuous stirring at 900 RPM on magnetic stirrer. The organic solvent was allowed to evaporate at room temperature on constant stirrer, resulting in formation of methylene blue loaded nanoparticles of PLGA. Further, the dispersion was centrifuge at 5000 RPM to separate the nanoparticles from the suspension and were lyophilized to obtain free flowing powder.
Preparation of coumarin loaded nanoparticles

The coumarin loaded PLGA nanoparticles were prepared by method proposed by Vanya B et al. [14] The 1:10 proportion of coumarin: PLGA were dissolved in 5 mL of acetone. The solution was mixed with 10mL of aqueous poloxamer 188 solution. The resulting suspension was stirred along with heating at 60°C using a magnetic stirrer for 3 hours to remove the organic solvent from dispersion. Further, the suspension was filtrated through a “white tape” paper filter and lyophilized to get free flow powder.

Conjugation of glutathione with nanoparticles

Conjugation of glutathione with nanoparticles was carried out as per the methodology proposed by Acharya S et al [15]; whereby 60 mg of nanoparticles were added in 2.4 mL phosphate buffer (pH 4.0) followed by drop-wise addition of an equal volume of EDAC solution having concentration of 50 mg/mL. The resultant mixture was incubated at room temperature for 45 minutes. The un-reacted EDAC was removed by centrifugation and the nanoparticles were re-suspended in 2.4 mL phosphate buffer (pH 7.4). The same volume of glutathione solution having the concentration of 50 mg/mL was added to the dispersion containing the activated nanoparticles and the mixture was stirred gently at room temperature for 6 hours. The conjugated nanoparticles were centrifuged for 30 minutes at 15000 RPM to remove excess of glutathione. Finally the glutathione conjugated nanoparticles were lyophilized to get free flowing powder. Nanoparticles were prepared using a nanoprecipitation method and were coated with enough reduced glutathione to yield a 2% w/v coating on the nanoparticles.

Particle size measurement of nanoparticles

Particle size and particle size distribution was measured by Malvern’s zeta-sizer. The nanoparticles were dispersed in suitable solvent and sonicated to prevent the agglomeration. Later on, the particle size was characterized after appropriate dilutions with deionized water.

In-vitro study

The in-vitro permeation of glutathione conjugated nanoparticles of PLGA was performed through establishing a Transwell co-culture of C6 (rat astrocytoma) cells and RBE4 (rat brain endothelial) cells [12] as shown in Figure 1, whereby the cell culture was allowed to developed on both the sides of permeable support.

Figure 1: Representation of the Transwell apparatus set-up to investigate the in-vitro permeability.

The comparative trans-BBB permeation of 10 μM each of MB free drug solution, uncoated MB nanoparticle and 2% glutathione-coated MB nanoparticle was
carried out by taking readings at predetermined intervals (0, 3, 6 and 24 hours).

**Results**

**PLGA Nanoparticles**

Experimental variations like process related parameters (i.e. stirring speed, rate of addition, evaporation rate, evaporation temperature, centrifugal force, incubation time, etc.) as well as the formulation parameters (i.e. concentration of polymers; type, volume and ratio of organic solvent; etc.) have shown to have the significant effect on the formulation and development of nanoparticles.

**Particle Size measurement**

Mean diameter of glutathione conjugated methylene blue loaded nanoparticle was found to be 220 nm, whereas glutathione conjugated coumarin loaded nanoparticle was 250 nm. Moreover, the particles size distribution for the glutathione conjugated methylene blue loaded nanoparticle was ranging between 190 mm to 250 nm, whereas glutathione conjugated coumarin loaded nanoparticle was between 200 nm to 300 nm.

**In-vitro study**

In-vitro study shown that the glutathione conjugated nanoparticles showed a significant increase in their trans-BBB permeation over 24 hours, while the free drug solution showed almost nil permeation across the Transwell system (Figure 2). Similar result pattern was observed for both the types of nanoparticles.

![Figure 2: Trans-BBB permeation of MB drug solution as compared to MB-loaded, glutathione-coated nanoparticles after 24 hours of treatment in Transwell apparatus.](image)

**Discussion**

**PLGA Nanoparticles**

The results revealed that formulation of nanoparticle is very complicated process and variety of process related parameters and the formulation related parameters significantly affects the physico-chemical properties of nanoparticles, which in turn affect the therapeutic outcome of the nanoparticles. Hence, careful design and optimization of each variable is essential to achieve desired properties of nanoparticles.

**Particle size measurement**

Experimental variables like amount of polymer, amount of surfactant content and amount of drug affect significantly to particle size distribution. The amount of
PLGA content is directly proportional to particle size diameter. According to Quintanar Guerrero et al. [16] study of PLGA content influence on nanoparticle size distribution, which suggest that as higher polymer amount promotes the aggregation due to less repulsive force between two particles, leading to increase in the mean particle size by aggregating with each other. The drug loading also showed direct relation with particle size distribution, due to higher entrapment efficiency of carrier and free drug tries to absorb on surface of nanoparticle causing aggregate [17]. Glutathione content also increases bulkiness of particle after conjugation, therefore, over-time of conjugation increases particle size [18].

In-vitro study

The in-vitro study of the glutathione conjugated coumarin loaded nanoparticles shown significant tran-BBB permeation as compare to unconjugated nanoparticle, which suggest that p-gp transporter efflux-out unconjugated nanoparticle successfully; however, the glutathione conjugated nanoparticles are able to cross the BBB. Overall uptake study reveals the vital role of glutathione as a carrier for trans-BBB permeation. Therefore glutathione may serve as an better vehicle for paclitaxel to deliver it in brain for the cancer treatment.

Conclusion

The in-vitro permeation study reveals that the glutathione conjugated nanoparticles of PLGA are able to cross the BBB. The success of this glutathione method should be investigated first in-vivo in animal study and then in human subjects to determine its clinical efficacy. This newer approach may provide a valuable therapeutic outcome and may provide the biomedical benefits in the number of therapies that are targeted to the brain and facilitates reduction of the morbidity faced by patients with CNS malignancies.

References


