RESEARCH PAPER

Design and Characterization of Folate-Modified Dual-Drug-Loaded Exosome-Based Nanocarriers for Targeted Glioblastoma Delivery

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Abstract

Background/Aim: Glioblastoma multiforme (GBM) is an aggressive primary brain tumor with poor therapeutic results due to poor drug permeability across the blood–brain barrier (BBB) and systemic toxicity of conventional chemotherapy. This study aims to develop and characterize folate-modified exosome-based nanocarriers co-loaded with berberine and temozolomide for targeted delivery to glioblastoma.

Methodology: Exosomes generated from adipose-derived mesenchymal stem cells were used as nanocarriers for dual-drug co-delivery via sonication-assisted encapsulation, and the exosomes were further modified to bear folate on their surface via EDC/NHS coupling chemistry. The nanoformulations were examined for particle size, PDI, zeta potential, morphology, encapsulation efficiency, drug loading, FTIR, in vitro drug release, stability, cytotoxicity, and BBB permeability.

Results: The developed BBR-TMZ-Exo-FA formulation exhibited a particle size of 134.2 nm, PDI of 0.237, encapsulation efficiency of 87.97%, and drug loading of 42.32%. FTIR suggested successful folate conjugation and dual-drug encapsulation. Sustained pH-responsive drug release was observed with maximum release at acidic pH 4.6. Enhanced cytotoxicity was observed compared with other formulations with cell viability of 28.42% and 27.55% in U87MG and U251MG cells, respectively, alongside enhanced BBB permeability (Papp: 7.03×10^{-6} cm/s) and physicochemical stability over 90 days.

Conclusion: The developed folate-modified dual-drug-loaded exosomal nanocarriers demonstrated favorable physicochemical properties, enhanced targeted cytotoxicity, and improved BBB permeation, representing a promising platform for targeted glioblastoma therapy and warranting further in vivo investigation.

Keywords: Berberine, Blood–brain barrier (BBB) targeting, Dual-drug delivery, Exosome-based nanocarriers, Folate receptor targeting, Glioblastoma, Temozolomide.

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1. Introduction

Glioblastoma multiforme (GBM) is one of the most aggressive and invasive primary brain malignancies, characterized by rapid proliferation, extensive cellular heterogeneity, and diffuse infiltration into surrounding brain tissue, resulting in a poor clinical prognosis and limited survival [1]. Despite advances in neurosurgical techniques and multimodal therapeutic approaches, the standard treatment regimen comprising surgical resection followed by radiotherapy and temozolomide (TMZ)-based chemotherapy provides only limited survival benefits, with a median survival duration of approximately 15 months [2]. The poor therapeutic efficacy is primarily associated with inadequate drug accumulation within brain tissues, nonspecific systemic distribution, and restricted penetration across the blood–brain barrier (BBB) [3].

The BBB represents a highly selective physiological barrier that significantly restricts the transport of therapeutic agents into the central nervous system, thereby limiting the effectiveness of conventional chemotherapeutic agents against glioblastoma [4]. Furthermore, the nonspecific biodistribution of anticancer drugs contributes to systemic toxicity and reduced therapeutic concentration at the tumor site [5]. Therefore, the development of efficient brain-targeted drug delivery systems that improve BBB permeation and enhance localized drug accumulation remains a major challenge in glioblastoma therapy.

Nanotechnology-based drug delivery systems have emerged as promising approaches to overcome the limitations of conventional chemotherapy by improving drug stability, bioavailability, circulation time, and targeted delivery efficiency [6]. Among these, exosome-

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based nanocarriers have attracted considerable attention owing to their intrinsic biocompatibility, low immunogenicity, nanoscale dimensions, and inherent capability to traverse biological barriers, including the BBB [7]. Exosomes are naturally occurring extracellular vesicles that facilitate intercellular communication and have significant potential as drug-delivery vehicles for central nervous system disorders [8].

Surface functionalization strategies have further enhanced the targeting efficiency of exosome-based delivery systems. Folate receptor-mediated targeting has been widely explored because folate receptors are overexpressed in various malignant cells, including glioblastoma, thereby facilitating enhanced cellular uptake and targeted drug delivery [9]. Functionalization of exosomal surfaces with folate ligands may therefore improve BBB permeation and increase drug accumulation within glioblastoma tissues.

Temozolomide (TMZ) remains the first-line chemotherapeutic agent for glioblastoma management; however, its clinical efficacy is often limited by inadequate intracellular delivery and poor brain bioavailability [10]. Berberine, a naturally occurring isoquinoline alkaloid, has demonstrated promising anticancer activity in glioma models and has been investigated for its potential therapeutic applications in brain malignancies [11]. Therefore, the co-delivery of berberine and TMZ using exosome-based nanocarriers may provide an effective strategy to improve targeted glioblastoma delivery and enhance drug transport across the BBB.

Despite recent advancements in nanocarrier-mediated brain delivery systems, the development of stable and surface-functionalized exosomal formulations capable of efficient dual-drug delivery remains limited. Although folate-functionalized exosomal systems have been explored for glioblastoma therapy, the co-delivery of berberine and temozolomide using ADSC-derived exosomes has received limited attention. Therefore, the present study aimed to design and characterize folate-modified exosome-based nanocarriers co-loaded with berberine and temozolomide for targeted glioblastoma delivery. The developed nanoformulations were further evaluated for their physicochemical characteristics, drug loading efficiency, release behavior, stability, cytotoxicity, cellular uptake, and blood–brain barrier (BBB) permeation potential.

2. Methodology

2.1. Study design

The present study was designed as an experimental laboratory-based investigation involving formulation optimization, physicochemical characterization, in vitro drug release, stability evaluation, preliminary cytotoxicity assessment, cellular uptake analysis, and blood–brain barrier penetration studies of folate-modified dual-drug-loaded exosomal nanocarriers for targeted glioblastoma delivery.

2.2. Study setting

The study was conducted in a specialized pharmaceutical nanotechnology and cell culture research laboratory equipped for formulation development, physicochemical characterization, and in vitro evaluation of exosome-based nanoformulations.

2.3. Materials and Chemicals/Reagents

Table 1: Materials and Chemicals/Reagents used [12].

Category	Material / Chemical	Source (Supplier)
Drugs	Berberine	Sigma-Aldrich, USA
	Temozolomide	Sigma-Aldrich, USA
Exosome Source	Mesenchymal Stem Cells (MSCs)	ATCC, USA
Chemicals/Reagents	Phosphate Buffered Saline (PBS)	HiMedia, India
	Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher, India
	Fetal Bovine Serum (FBS)	Thermo Fisher, India
	Dimethyl Sulfoxide (DMSO)	Merck, Germany
	Methanol	Merck, Germany
	MTT Reagent	Sigma-Aldrich, USA
	Dialysis Membrane	HiMedia / Sigma-Aldrich
	Exosome Isolation Reagents	Invitrogen / Thermo Fisher
	Folic Acid	Sigma-Aldrich, USA
	N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC)	Thermo Fisher Scientific, USA
	N-Hydroxysuccinimide (NHS)	Thermo Fisher Scientific, USA

2.4. Isolation and Purification of Exosomes

Exosomes were isolated from conditioned media of adipose-derived mesenchymal stem cells (ADSCs) owing to their high biocompatibility, immunomodulatory properties, and suitability as nanocarriers for brain-targeted drug delivery. ADSCs were cultured in DMEM supplemented with 10% exosome-depleted fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2. Cells between passages 3–5 were used, and conditioned medium was collected after 48 h of culture. Initially, the conditioned medium was centrifuged at 300–500 × g for 10 min to remove intact cells, followed by centrifugation at 2,000 × g for 20 min to eliminate dead cells and

cellular debris. Subsequently, the supernatant was centrifuged at 10,000 × g for 30 minutes to remove larger extracellular vesicles. The resulting supernatant was filtered through a 0.22 μm membrane filter and subjected to ultracentrifugation at 100,000 × g for 60–90 minutes for exosome isolation. The isolated exosome pellets were washed with phosphate-buffered saline (PBS) and re-ultracentrifuged under identical conditions to improve purity. Purified exosomes were resuspended in PBS and stored at −80°C until further use. The isolated exosomes were characterized using exosomal surface markers CD9, CD63, and CD81.

2.5. Incorporation of Drugs into Exosomes

Berberine and temozolomide were encapsulated into isolated exosomes via sonication-assisted encapsulation. Briefly, both drugs were prepared individually at 50 μM and incubated with exosome suspensions (100 μg/mL) under optimized conditions. The pH of the formulation medium was adjusted to 5.5–6.5 using citrate buffer to facilitate drug–exosome interaction. The mixture was subjected to six sonication cycles of 3 minutes each with intermittent cooling, followed by incubation at 37°C for 60 minutes to stabilize membrane resealing and improve drug entrapment efficiency. Subsequently, unencapsulated drugs were removed by ultracentrifugation at 100,000 × g for 60–90 minutes, followed by purification using 100 kDa centrifugal filtration to obtain purified dual-drug-loaded exosomes [12].

2.6. Surface Modification of Exosomes

Surface functionalization of the exosomal nanocarriers was performed to enhance BBB permeation and glioblastoma-targeted delivery. Folic acid was selected as the targeting ligand owing to its affinity toward folate receptors overexpressed in glioblastoma and brain endothelial cells. Conjugation was performed using carbodiimide chemistry involving 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) [13]. Briefly, folic acid was dissolved in dimethyl sulfoxide (DMSO) and activated using EDC/NHS before conjugation with exosomal membrane surface groups [12]. Folic acid (2 mg/mL) was activated using EDC (4 mg/mL) and NHS (6 mg/mL) prior to conjugation with exosomal membrane surface groups under continuous stirring at room temperature. The reaction facilitated stable covalent linkage between folate ligands and exosomal surface moieties without compromising the structural integrity of the nanoformulations.

2.7. Characterization of Formulation

2.7.1 Particle Size, PDI, and Zeta Potential

The physicochemical analysis of the nanoformulations was conducted by dynamic light scattering (DLS) using a Zetasizer from Beckman Coulter, in which particle size, polydispersity index (PDI), and zeta potential were determined. DLS was used to determine hydrodynamic diameter, PDI, and surface charge. Before performing the measurements, all samples were appropriately diluted with deionized water, and the pH was maintained between 6.8 and 7.0. To avoid aggregation and achieve proper dispersion of the sample particles, they were sonicated for 5 minutes before analysis [14]. All measurements were performed in triplicate, and the results were expressed as mean ± standard deviation (SD).

2.7.2 Morphological Analysis

The Morphological characterization of the nanocarriers was performed using a Scanning Electron Microscope (SEM) and a Transmission Electron Microscope (TEM). SEM characterization of the samples was performed by loading the sample solution onto a glass coverslip, drying it, sputtering with gold, and analyzing it with a JEOL JSM-6380LV (Japan). Transmission electron microscopy analysis of the samples was performed by mounting them on a carbon-coated copper grid and staining them with 2% uranyl acetate [12].

2.7.3 Encapsulation Efficiency and Drug Loading

Encapsulation efficiency of TMZ-loaded exosomes was calculated by isolating free TMZ from exosome-bound TMZ using centrifugation through an Amicon Ultra-100kDa filter. Free TMZ was measured by UV-visible spectroscopy at 370 nm using a previously prepared standard calibration curve of TMZ solution. The entrapment efficiency of the drug was determined by subtracting the drug that was free from the total quantity of drug loaded when formulating. The efficiency of encapsulation was given as the percentage of the drug trapped in the exosomal formulation divided by the total quantity of drug used [12]:

$$(\%)\ \boldsymbol{Entrapment\ Efficiency} = \frac{\boldsymbol{Total\ drug\ added - Free\ drug}}{\boldsymbol{Total\ drug\ added}} \times \mathbf{100}$$

The amount of drug loading for TMZ with exosomes was obtained by determining the quantity of the drug entrapped in the exosome carriers in relation to the weight of the entire exosome sample. Following filtration to separate the non-entrapped drug using Amicon Ultra-100 kDa centrifugal filters, the entrapment drug quantity was determined by UV–Visible spectrophotometry at 370 nm, using a calibration curve. The percentage of drug loading for the drug was determined based on the total amount of weight of the entire exosome sample, as calculated below [12]:

$$(\%)\ \boldsymbol{Drug\ Loading\ Efficiency} = \frac{\boldsymbol{Amount\ of\ drug\ encapsulated}}{\boldsymbol{Total\ weight\ of\ nanocarriers}} \times \mathbf{100}$$

Similarly, the encapsulation efficiency and drug loading of berberine were determined by measuring the concentration of unencapsulated berberine in the filtrate following centrifugal separation using Amicon Ultra-100 kDa filters. Berberine concentration was quantified by UV–Visible spectrophotometry at 418 nm using a previously established calibration curve. The encapsulation efficiency and drug loading were calculated using the same equations employed for temozolomide.

2.7.4 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Fourier transform infrared spectroscopy (FTIR) analysis was performed using a PerkinElmer FTIR spectrometer (USA) to evaluate drug–excipient compatibility and confirm successful folate conjugation on exosomal surfaces. Lyophilized samples were mixed with potassium bromide (KBr), compressed into pellets, and scanned within the spectral range of 4000–400 cm^{-1} [12].

2.8. In Vitro Drug Release Study

The in vitro kinetic studies on the release of berberine (BBR) and Temozolomide (TMZ) from surface-modified exosome-based drug delivery systems were conducted by employing dialysis bag diffusion techniques. Briefly, the loaded exosomes (BBR-Exo), TMZ-Exo, BBR-TMZ-Exo, and BBR-TMZ-Exo-FA were introduced into dialysis bags (12 kDa molecular weight cutoff) submerged in phosphate buffer solutions containing 20% v/v ethanol at pH 4.6, 5.0, 6.5, and 7.4, respectively. Samples were taken at preset time intervals (0–96 h) and replenished with fresh buffer to ensure sink conditions. The amounts of BBR and TMZ released were measured by UV-Visible spectrophotometry at 418 nm and 370 nm, respectively [12,15]. The selected pH conditions were used to simulate different physiological environments. pH 4.6 represented endosomal/lysosomal acidic conditions, pH 6.5 mimicked the tumor microenvironment, and pH 7.4 represented physiological conditions.

$$\boldsymbol{Released}\ (\%) = \frac{[\boldsymbol{Drug}]_{\boldsymbol{rel}}}{[\boldsymbol{Drug}]_{\boldsymbol{load}}} \times \mathbf{100}$$

2.9. Stability Studies of Formulated Nanocarriers

Stability studies of the developed nanoformulations were conducted at refrigerated (4°C) and room-temperature (25°C) storage conditions. The formulations were periodically evaluated for changes in particle size, PDI, zeta potential, drug release profile, and physical appearance. Accelerated stability testing was also performed using heating–cooling cycles (45 ± 2°C to 25 ± 2°C) and freeze–thaw cycles (−20 ± 2°C to 25 ± 2°C), repeated six times with 24-hour intervals at each temperature. Stability assessment was performed in accordance with the applicable ICH stability evaluation considerations [16].

2.10. In Vitro Cytotoxicity Assessment

Preliminary cytotoxicity evaluation of the developed nanoformulations was performed using U87MG and U251MG glioblastoma cell lines cultured in high-glucose DMEM and DMEM/F-12 media supplemented with 10% fetal bovine serum, respectively. Cells were seeded at a density of 1.5×10^4 cells/well in 96-well plates and incubated for 24 hours before treatment. Experimental groups included untreated control cells, empty exosomes, free drugs, single-drug-loaded exosomes, dual-drug-loaded exosomes, and folate-functionalized nanoformulations. Cells were treated with formulations equivalent to 100 µg/mL total drug concentration and incubated for 48 h under standard culture conditions. After 48 hours of treatment, cell viability was evaluated using the MTT assay (0.5 mg/mL). Formazan crystals formed were dissolved in DMSO, and absorbance was measured at 570 nm using a microplate reader [12].

2.11. Cellular Uptake Assessment

Cellular uptake of dye-labeled exosome-based nanoformulations was analyzed using confocal laser scanning microscopy (CLSM). Glioblastoma cells cultured on coverslips were incubated with fluorescently labeled nanoformulations for predetermined durations, followed by PBS washes to remove excess non-internalized nanoparticles. Cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 1% bovine serum albumin (BSA). Nuclei were stained using Hoechst 33342, while filamentous actin was stained with Alexa Fluor® 647 phalloidin. The stained samples were mounted using ProLong Gold Antifade reagent and visualized using a Nikon A1R confocal microscope at appropriate excitation wavelengths [17].

2.12. Blood–Brain Barrier (BBB) Penetration

The BBB permeability of the folate-functionalized exosome-based nano-carrier was assessed using an in vitro BBB co-culture model comprising brain endothelial cells, astrocytes, and pericytes. The integrity of the BBB model system was validated using trans-endothelial electrical resistance (TEER) analysis, in which BBB integrity is indicated by a resistance measurement within the range of 150-300 Ω·cm², signifying intact tight junctions. TEER values between 150 and 300 Ω·cm² were considered indicative of an intact BBB model and were confirmed prior to permeability assessment. The fluorescently labeled folate-functionalized exosome, non-folate-functionalized exosome, and drug-loaded free formulation were added to the apical side of the cell model and incubated for various time points. The samples collected from the basolateral side were quantified using a fluorescence spectrometer to measure the nanoparticle transport through the BBB. The P_{app} value was determined for assessing the permeation rate of nanoparticles. A higher permeation rate was expected for the folate-functionalized exosome group [18].

$$P_{app} = \frac{V}{A \times [C]_{apical}} \times \frac{\Delta[C]_{basolateral}}{\Delta t}$$

Where

P_{app}: Permeability coefficient

$[C]_{apical}$: initial concentration of fluorescent nanoparticles on the apical side

$\Delta[C]_{basolateral}$: differential concentration of fluorescent nanoparticles on the basolateral side

A: surface area of membrane

V: medium volume in the basolateral side.

2.13. Statistical analysis

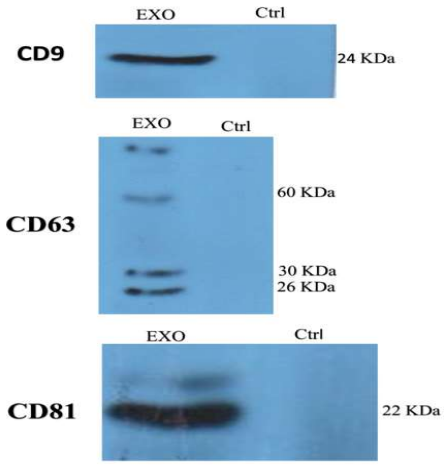
Statistical analyses were performed using IBM SPSS Statistics 26 and GraphPad Prism software. Experimental data were represented as mean ± standard deviation (SD) obtained from three independent experiments. One-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test was applied for multiple-group comparisons, while Student's t-test was used for pairwise analysis. A p-value of less than 0.05 was considered statistically significant.

3. Results

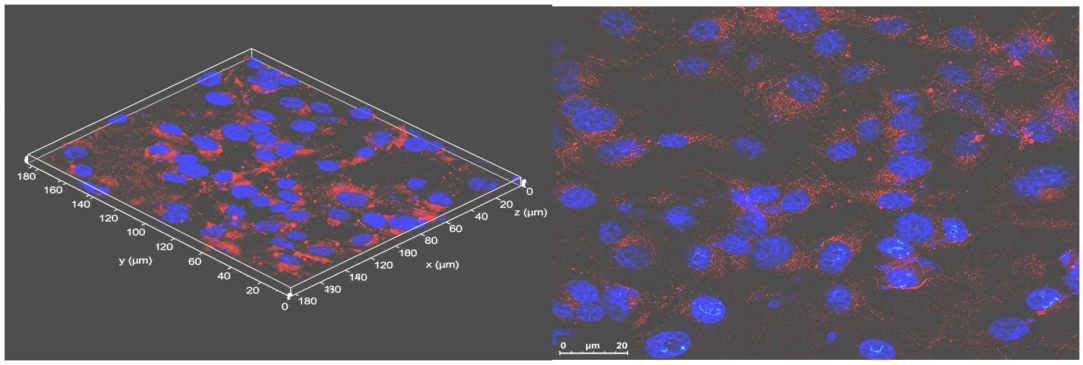
3.1 Preparation of Folate-Modified Dual-Drug-Loaded Exosomal Nanocarriers

3.1.1 Isolation and Purification of Exosomes

ADSCs-derived exosomes were successfully recovered and purified by differential centrifugation and ultracentrifugation. The consecutive centrifugation processes effectively eliminated cells, cellular detritus, and larger extracellular vesicles, resulting in a pure exosome fraction. The effective extraction of exosomes appropriate for future formulation development was validated by the presence of exosomal markers CD9, CD63, and CD81 (Figure 1a-1c).



(a)



(b) (c)

Figure 1: Characterization of ADSCs-Derived Exosomes Showing (a) Western Blot Analysis of Exosomal Markers CD9, CD63, and CD81, (b) 3D Z-stack and (c) 2D CLSM Images of PKH-Labeled Exosomes (Red) Internalized in Glioblastoma Cells with DAPI-Stained Nuclei (Blue)

3.1.2 Incorporation of Berberine and Temozolomide into Exosomes

The sonication-assisted loading technique successfully loaded berberine and temozolomide into purified exosomes. The temporary membrane permeabilization induced by sonication enabled effective drug trapping in exosome vesicles. The resultant dual-drug-loaded exosome formulation was successfully prepared for further characterisation and assessment. This observation was further supported by encapsulation efficiency and drug loading data (Table 2).

3.1.3 Surface Functionalization with Folic Acid

The dual-drug-loaded exosomes was subjected to folic acid surface modification using EDC/NHS-induced carbodiimide coupling. The conjugation method maintained the structural stability of the nanocarriers while incorporating a target ligand for glioblastoma-specific delivery. The obtained folate-functionalized

exosome formulation was then used for physicochemical characterisation and biological research (Figure 2).

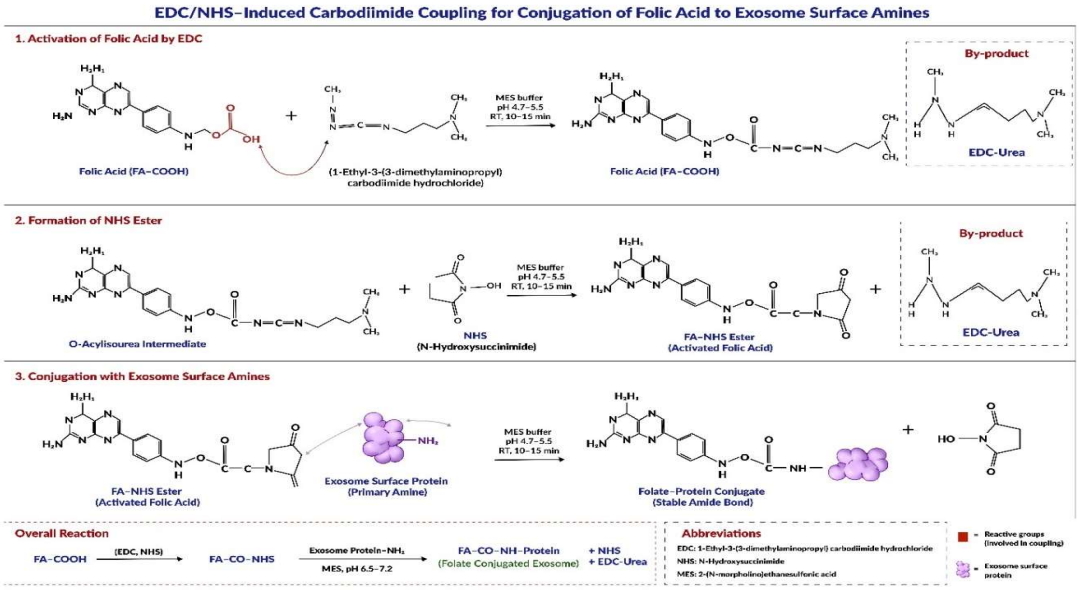


Figure 2: EDC/NHS-mediated carbodiimide coupling chemistry for covalent conjugation of folic acid to exosomal surface amine groups

3.2 Characterization of Formulation

3.2.1 Particle Size, (PDI), and Zeta Potential

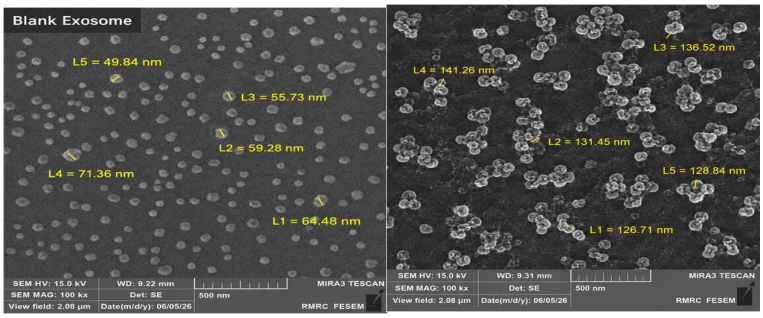
The particle size, PDI, and zeta potential of the produced exosomal formulations were analysed by DLS (Table 1). The average particle size of the blank exosomes was 99.4 nm and increased after drug loading along with folate conjugation. The intended formulation (BBR-TMZ-Exo-FA) exhibited the maximum particle size (134.2 nm), suggesting successful drug incorporation and surface modification. The PDI values of all the formulations were less than 0.3, indicating a narrow size distribution and acceptable homogeneity. All formulations exhibited negative Zeta potential, supporting the colloidal stability of the exosomal nanocarriers.

Table 1: Physicochemical Properties (Particle Size, PDI, and Zeta Potential) of Exosomal Nanoformulations

Formulations	Particle Size (nm)	PDI	Zeta Potential (mV)
Blank Exosome	99.4 ± 1.433	0.177 ± 0.0008	-23.5 ± 1.134
BBR-Exo	112.0 ± 3.706	0.213 ± 0.0009	-18.4 ± 1.986
TMZ-Exo	108.9 ± 4.509	0.186 ± 0.0012	-19.4 ± 0.124
BBR-TMZ-Exo (dual)	124.7 ± 3.080	0.219 ± 0.0194	-20.4 ± 3.440
BBR-TMZ-Exo-FA (targeted)	134.2 ± 4.035	0.237 ± 0.0159	-15.8 ± 1.080

3.2.2 Morphological Analysis

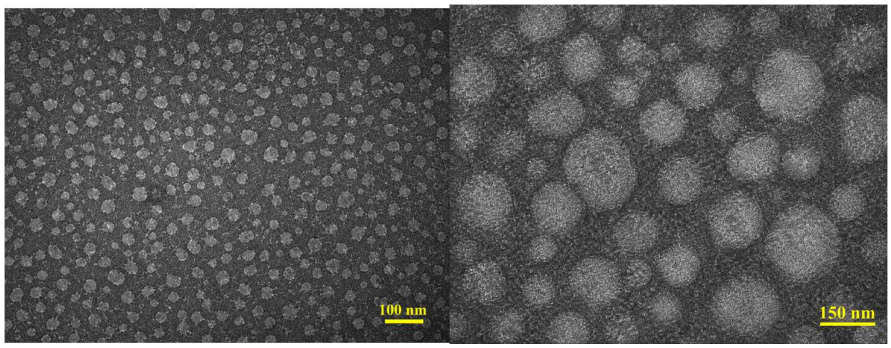
The scanning electron microscope characterized the spherical shape and surface structures of the nanoparticulate exosomal delivery systems. Blank exosomes (Fig. 3a) possessed evenly dispersed nanoparticles with particle sizes of between 49.84 and 71.36 nm. In comparison, drug-loaded exosomes functionalized with folates (BBR-TMZ-Exo-FA) (Fig. 3b) showed significantly larger particles with particle sizes of between 126.71 and 141.26 nm in slight aggregates, which confirmed successful loading of the two drugs and surface modifications without altering the spherical structure.



(a) (b)

Figure 3: SEM Images of (a) Blank Exosomes and (b) BBR-TMZ-Exo-FA Nanocarriers

The nanoscale dimensions and intact vesicular structure of the exosomal nanocarriers were further confirmed by transmission electron microscopy. The blank exosomes (Figure 4a) showed the appearance of uniformly distributed, electron-dense spherical nanoparticles in the 100 nm scale, while the drug-loaded formulation (Figure 4b) showed larger, distinctly membrane-bound vesicular structures in the 150 nm scale, with characteristic cup-shaped morphology consistent with exosomal architecture. The increase in particle size after drug loading and surface modification confirmed the DLS results and validated the successful formulation development.



(a) (b)

Figure 4: TEM Images of (a) Blank Exosomes and (b) BBR-TMZ-Exo-FA Nanocarriers

The smaller particle sizes detected via SEM as opposed to DLS can be due to the dehydration of the samples prior to scanning, while DLS determines the hydrodynamic diameter of the suspended particles.

3.2.3 Encapsulation Efficiency and Drug Loading

Encapsulation efficiency and drug loading of exosome formulations demonstrated a progressive enhancement following drug incorporation and surface modification (Table 2). The blank exosomes exhibited baseline loading capacity, while BBR-Exo and TMZ-Exo showed moderate and comparable encapsulation efficiencies of 74.36% and 72.37%, respectively, indicating efficient individual drug incorporation within the vesicular structure.

The dual drug-loaded formulation (BBR-TMZ-Exo) demonstrated a noticeable increase in EE (79.79%) and DL (33.89%), indicating a synergistic effect of co-loading without affecting the vesicle integrity. Further increase was seen after surface functionalization with folic acid (BBR-TMZ-Exo-FA), which showed the best encapsulation efficiency (87.97%) and drug loading (42.32%), suggesting improved drug retention within the exosomal carrier system perhaps due to ligand-mediated surface interactions.

Table 2: Encapsulation Efficiency and Drug Loading of Exosome Formulations

Formulation	EE (%)	DL (%)
BBR-Exo	74.36 ± 0.31	29.62 ± 0.24
TMZ-Exo	72.37 ± 0.68	27.27 ± 0.30
BBR-TMZ-Exo	79.79 ± 0.21	33.89 ± 0.30
BBR-TMZ-Exo-FA	87.97 ± 0.39	42.32 ± 0.29

3.3 Surface Modification Analysis

The conjugation of folic acid (FA) on the surface of the exosomes was confirmed by FTIR analysis. Purified exosomes were lyophilized and analyzed by the KBr pellet technique for the identification of distinctive functional groups and bond

formation (Figure 5). The blank exosome spectrum showed characteristic peaks at 3441 and 2932 cm-1 corresponding to O-H/N-H and C-H stretching vibrations, respectively. The BBR-TMZ-Exo-FA formulation showed additional peaks at 1651 cm^{-1} and 1436 cm^{-1}, which were attributed to C=O stretching and C–H bending vibrations of folic acid and drug-associated absorption bands, suggesting the successful dual-drug encapsulation. A significant change in the C–O stretching vibrations in the 900–1052 cm^{-1} range suggested the formation of stable covalent bonds between the folate ligand and exosomal surface moieties using EDC/NHS carbodiimide coupling chemistry.

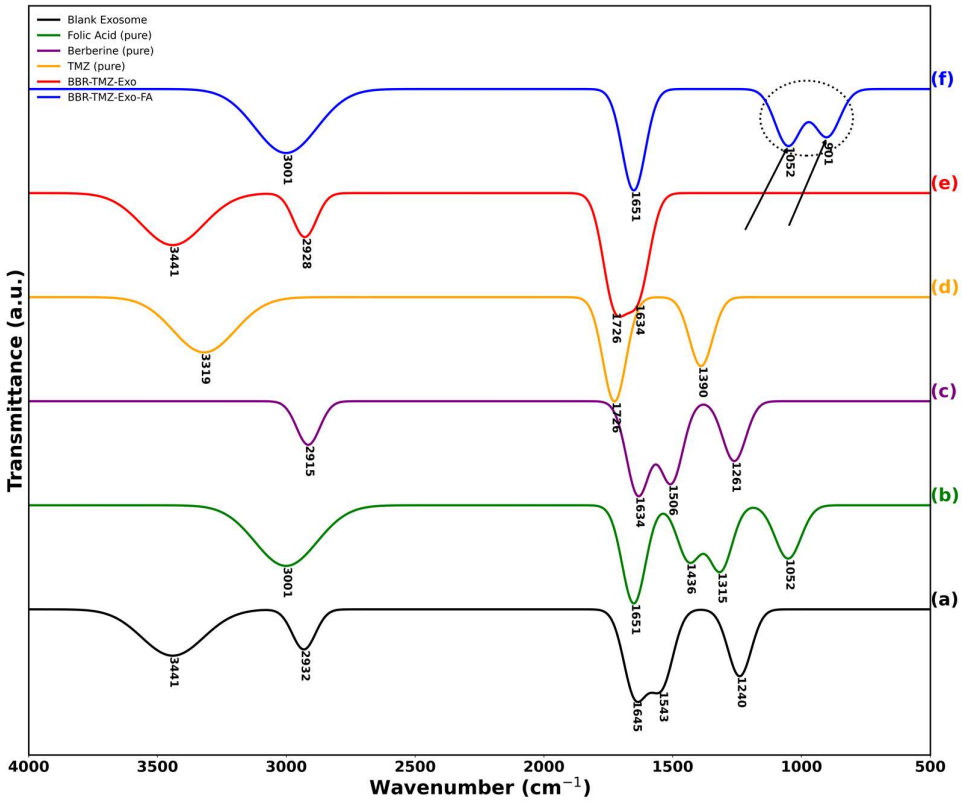


Figure 5: FTIR Spectra of Exosomal Formulations Confirming Drug Encapsulation and Folate Conjugation

3.4 In Vitro Drug Release Study

The In vitro release study showed that all the exosome formulations showed a sustained and pH-dependent drug release pattern for all four formulations over 96 hours (Figure 6). All the formulations exhibited maximum cumulative drug release at pH 4.6 (acidic) (~ 90–100 % at 96 h), which was gradually decreased at pH 5.0, 6.5, and physiological pH 7.4, confirming pH-responsive behaviour. The folate-functionalized dual-drug-loaded formulation (BBR-TMZ-Exo-FA) showed the most significant release profile at acidic pH, and sustained and controlled release pattern at physiological pH, indicating enhanced release under acidic conditions while maintaining systemic stability. The enhanced release characteristics in acidic conditions and the slow release at physiological pH implied better drug availability at the tumour site with stability in systemic circulation. The folate-modified dual-drug-loaded exosomes were found to be suitable for potential glioblastoma-targeted drug delivery applications.

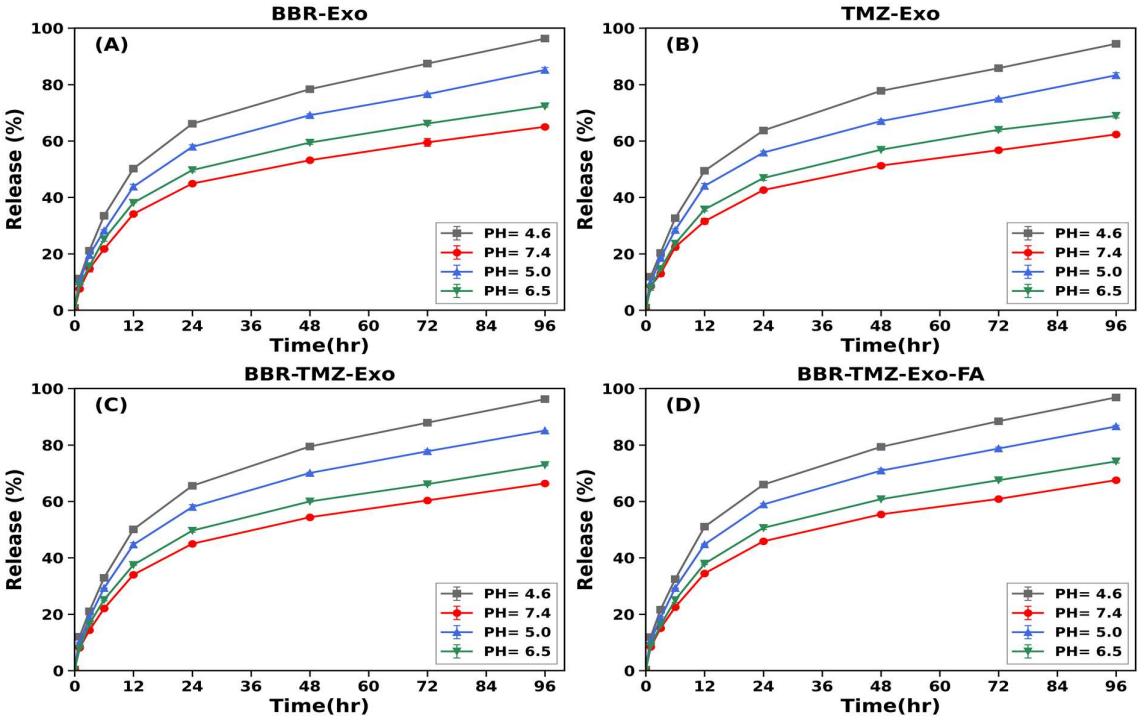


Figure 6: In Vitro Cumulative Drug Release (%) of Exosome Formulations at Different pH Conditions

3.5 Stability Study

The produced exosome formulations exhibited good physicochemical stabilities for 90 days under both storage conditions (Table 3). There was only a small change in particle size. The PDI values were less than 0.26, showing that the homogeneity of the size distribution was maintained and there was no substantial aggregation seen. The zeta potential values were slightly modified, but still negative enough for colloidal stability. The folate-modified formulation was as stable as the non-targeted formulation, suggesting that surface functionalization did not affect the integrity of the vesicles. Overall, the formulations stored at 4 °C were more stable than those stored at 25 °C, with less change in the size of particles and surface charge. The research period did not show any visible alterations in the morphology, the precipitation pattern, and the separation of phases, which supports the potential of the developed exosomal nanocarriers for long-term storage and for selective glioblastoma-drug delivery applications.

Table 3: Stability Profile of Dual-Drug-Loaded Exosome Formulations During 90-Day Storage

Formulations	Days	Storage (4°C)			Storage (25°C)		
		Particle Size (nm)	PDI	Zeta Potential (mV)	Particle Size (nm)	PDI	Zeta Potential (mV)
BBR-TMZ-Exo	0	123.63 ± 1.04	0.238 ± 0.011	-18.37 ± 0.46	124.30 ± 0.73	0.219 ± 0.010	-19.07 ± 0.54
	7	124.73 ± 0.90	0.228 ± 0.002	-19.00 ± 0.96	125.43 ± 1.11	0.229 ± 0.003	-17.97 ± 0.34
	14	125.57 ± 1.44	0.234 ± 0.011	-17.70 ± 0.59	125.00 ± 0.70	0.233 ± 0.009	-17.80 ± 0.78
	30	126.40 ± 0.51	0.229 ± 0.003	-17.27 ± 0.97	126.97 ± 0.74	0.227 ± 0.000	-17.70 ± 0.43
	60	128.13 ± 0.94	0.238 ± 0.005	-17.60 ± 1.14	130.63 ± 0.54	0.241 ± 0.015	-18.13 ± 0.82
	90	131.07 ± 1.96	0.230 ± 0.006	-17.33 ± 0.59	135.07 ± 1.45	0.250 ± 0.001	-16.37 ± 1.39
BBR-TMZ-Exo-FA	0	134.10 ± 1.42	0.240 ± 0.005	-17.40 ± 1.04	134.37 ± 0.52	0.237 ± 0.013	-17.23 ± 0.39
	7	135.53 ± 1.70	0.242 ± 0.005	-16.40 ± 0.45	134.40 ± 0.57	0.232 ± 0.003	-17.77 ± 0.17
	14	136.20 ± 0.37	0.250 ± 0.007	-16.47 ± 0.73	136.70 ± 0.57	0.240 ± 0.014	-16.77 ± 0.34

	30	136.90 ± 1.49	0.248 ± 0.003	-16.07 ± 0.74	137.70 ± 1.35	0.242 ± 0.007	-16.67 ± 0.49
	60	138.93 ± 0.60	0.246 ± 0.007	-16.17 ± 1.11	142.53 ± 2.33	0.257 ± 0.007	-16.37 ± 0.12
	90	141.33 ± 0.71	0.245 ± 0.005	-14.97 ± 0.05	146.03 ± 0.61	0.254 ± 0.010	-15.00 ± 0.29

***Data are expressed as mean ± SD (n = 3).**

3.6 Preliminary Cytotoxicity Assessment

MTT experiment indicated the decrease in cell viability in both the U87MG and U251MG glioma cell lines in a formulation-dependent way ($p < 0.0001$). The empty exosomes were shown to be intrinsically biocompatible (cell viability >94%). Still, the drug-loaded exosomal formulations showed a greater cytotoxicity relative to the respective free drug formulations, suggesting an improved intracellular drug delivery via the exosomal carrier system. The folate-functionalized dual-drug-loaded formulation (BBR-TMZ-Exo-FA) showed the strongest antiproliferative activity, decreasing the cell viability to 28.42% and 27.55% in U87MG and U251MG cells, respectively, which may be attributed to enhanced cellular uptake through folate receptor mediated interactions suggesting its potential for targeted glioblastoma treatment (Figure 7).

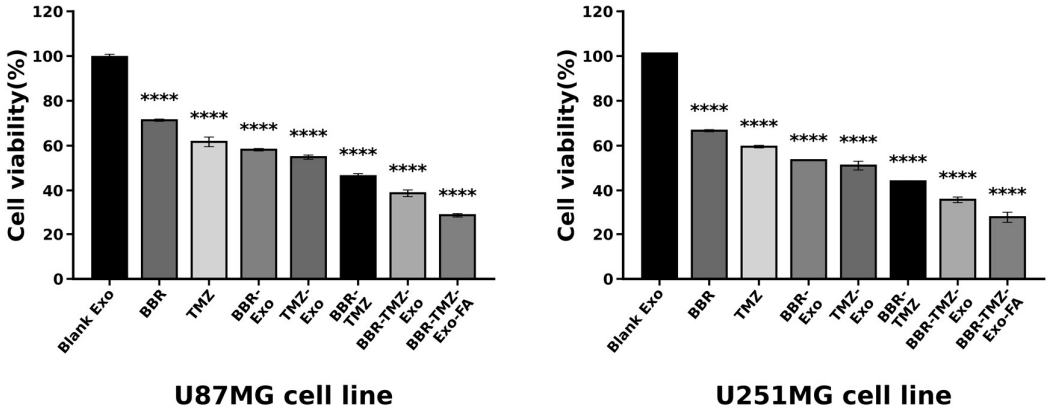


Figure 7: Cell Viability (%) of Exosome Formulations Against U87MG and U251MG Glioblastoma Cell Lines by MTT Assay

3.7 Cellular Uptake Study

Confocal laser scanning microscopy (CLSM) demonstrated increased cellular uptake of the exosome-based formulations in U87MG and U251MG cells. Free drug formulations showed little to no intracellular fluorescence, whereas exosome-encapsulated drugs showed much higher uptake. The dual drug-loaded exosomes showed higher fluorescence intensity than single drug-loaded formulations. Folate-functionalized dual-drug-loaded exosomes exhibited the highest intracellular accumulation among all groups, suggesting improved receptor-mediated endocytosis and efficient targeting to glioblastoma cells (Figure 8).

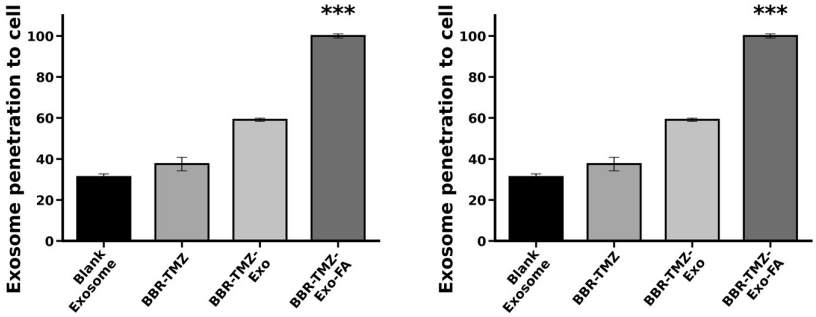


Figure 8: Cellular Uptake of Exosome-Based Formulations in U87MG and U251MG Glioblastoma Cells

3.8 BBB Penetration Efficiency

BBB penetration was assessed utilising an in vitro BBB co-culture model of brain endothelial cells, astrocytes, and pericytes (Table 4). Barrier integrity was established by TEER values (150–300 Ω·cm²). All formulations showed time-dependent BBB translocation (Table 6). Free drug demonstrated lowest permeability (2.16×10^{-6} cm/s at 6 h) while blank exosomes showed slightly

higher permeation (2.63 × 10-6 cm/s). Transport was improved in drug-loaded exosomes (BBR-TMZ-Exo) from 2.06 × 10−6 cm/s at 1 h to 4.13 × 10−6 cm/s at 6 h. The folate-functionalized formulation (BBR-TMZ-Exo-FA) showed the maximum permeability, reaching 7.03 × 10^{-6} cm/s at 6 h, which indicates the increased BBB transport and indicates enhanced BBB permeation potential compared with non-targeted formulations.

Table 4: In Vitro Blood–Brain Barrier Permeability of Exosomal Formulations Determined Using a BBB Co-Culture Model

Formulations	Hours (Papp ×10^{-6} cm/s)			
	1 h	2 h	4 h	6 h
Free Drug Solution	1.03 ± 0.10	1.35 ± 0.07	1.75 ± 0.15	2.16 ± 0.13
Blank Exosome	1.40 ± 0.06	1.68 ± 0.16	2.23 ± 0.02	2.63 ± 0.23
BBR-TMZ-Exo	2.06 ± 0.14	2.61 ± 0.07	3.37 ± 0.14	4.13 ± 0.06
BBR-TMZ-Exo-FA	3.36 ± 0.13	4.22 ± 0.03	5.74 ± 0.06	7.03 ± 0.08

4. Discussion

The present study developed folate-functionalized dual-drug-loaded exosomal nanocarriers for targeted glioblastoma therapy by integrating natural exosome-mediated transport and active ligand targeting to improve the therapeutic efficacy. The expression of CD9, CD63, and CD81 markers confirmed effective extraction of exosomes from ADSCs, confirming the vesicles were intact as well as biologically active for drug delivery applications. Similarly, the results by Maniya et al. (2024) confirmed a high expression of CD9, CD63, and CD81 in exosomes derived from glioblastoma, establishing this as a common feature of extracellular vesicles [19].

The sonication-assisted technique was successfully utilised to load berberine and temozolomide, where the membrane rupture and drug encapsulation are transient. In the same manner, Salarpour et al. (2019) found that sonication could remarkably enhance the drug loading and cytotoxic activity in U87 glioblastoma cells, supporting sonication as a competent exosome loading method [20]. The particle size increased from 99.4 nm (blank exosomes) to 134.2 nm after dual-drug loading and conjugation of folate without disturbing the nanoscale dispersion and stability. Likewise, Pourmasoumi et al. (2024) observed the sizes of the temozolomide–quercetin-loaded exosomes to be between 30 and 150 nm, indicating their potential for glioblastoma-targeted delivery and penetration of the blood–brain barrier [12].

FTIR analysis suggested successful folate conjugation and supported surface modification of the exosomal nanocarriers. Similarly, Pourmasoumi et al. (2024) experimentally revealed successful conjugation of folate to exosomal surfaces using chemical coupling techniques, which led to increased tumor-targeting capabilities and better cellular absorption in glioblastoma models [12]. The developed formulations were shown to be stable for 90 days without any significant change in particle size and without any agglomeration. Similarly, Kumeda et al. (2017) showed that exosomes' membrane integrity and structural stability were maintained after long-term storage at 4 °C [21].

In vitro release investigation indicated pH-responsive and sustained release of the drug. The drug release was significantly higher in acidic conditions than in physiological pH. Similarly, Kim et al. (2016) showed delayed release and improved intracellular delivery of paclitaxel using exosome-based nanocarriers, showing the promise of exosome-based nanocarriers in controlled drug delivery [22]. All exosomal formulations exhibited increased cellular absorption, and the dual drug-loaded, folate-functionalized exosomes had the highest intracellular accumulation through receptor-mediated endocytosis. Hao et al. (2024) showed that folate-conjugated exosomes enhanced intracellular drug delivery in glioma cells by promoting the internalisation via folate receptor-mediated endocytosis [23].

The increased cytotoxicity was attributed to the increased cellular uptake, and BBR-TMZ-Exo-FA was the most effective in decreasing the viability of glioblastoma cells. Likewise, Pourmasoumi et al. (2024) demonstrated that the folate-functionalized exosomes co-loaded with temozolomide and quercetin significantly enhanced cytotoxicity and reduced glioblastoma cells' viability compared to the free-drug formulations [12]. The folate-modified exosomes showed a significant increase in BBB permeability compared to the free medication formulation. Furthermore, Khatami et al. (2023) demonstrated that exosomes may cross the blood-brain barrier by receptor-mediated transcytosis without any artificial changes, which can aid in the effective delivery of medications to the brain [24].

Therefore, the new BBR-TMZ-Exo-FA formulation generally showed high drug loading efficiency, nanoscale size distribution, sustained release behaviour, better cellular uptake, improved cytotoxicity, and superior BBB permeability. Taken together, our findings are corroborated by previous experimental investigations revealing that designed and folate-functionalized exosomes greatly increase glioblastoma-targeted medication delivery. However, the study is confined to in vitro evaluation, and further in vivo pharmacokinetic, biodistribution, toxicity, and therapeutic efficacy studies are needed to evaluate clinical translational potential.

5. Conclusion

In conclusion, the current study successfully developed folate-modified exosomal nanocarriers co-loaded with

berberine and temozolomide for targeted glioblastoma delivery, possessing desirable physicochemical characteristics, high drug loading efficiency, and sustained and pH-responsive drug release. It enhanced cellular uptake with improved permeation through the blood–brain barrier. The results point out the scientific relevance of exosome-mediated nanocarrier systems with folate-based active targeting as a potential approach to enhance site-specific glioblastoma therapy and to overcome the limits of traditional drug delivery. However, additional in vivo pharmacokinetic, biodistribution, safety, and therapeutic efficacy investigations are required to confirm the translational potential of the system and to justify its future development towards clinical use in the management of brain tumors.

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