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Original Research Article

Preparation of N, N, N-trimethyl chitosan-functionalized retinoic acid-loaded lipid nanoparticles for enhanced drug delivery to glioblastoma

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Abstract

Purpose: *To formulate trimethyl chitosan-functionalized retinoic acid-encapsulated solid lipid nanoparticles for the effective treatment of glioma.*
Methods: *Retinoic acid-loaded solid lipid nanoparticles (R-SLNs) were prepared using homogenization followed by sonication. R-SLN surfaces were functionalized electrostatically with trimethyl chitosan as a nanocarrier (TR-SLNs) with enhanced anti-cancer activity. They were evaluated by dynamic light scattering (DLS), scanning electron microscopy, in vitro drug release, and cell cytotoxicity and apoptosis studies.*
Results: *Morphological images showed spherical and uniformly dispersed nanoparticles. A sustained monophasic release pattern was observed throughout the study period. Furthermore, the anti-cancer effect of TR-SLNs was demonstrated by increased cell killing activity compared with the free drug ($p < 0.01$); negligible cytotoxicity was observed with blank carriers. Apoptosis assay showed increased cell populations in early/late apoptotic and necrotic phases.*
Conclusion: *This study showed the potential application of surface-modified solid lipid nanoparticles for the effective treatment of brain cancer.*

Keywords: *Lipid nanoparticles, Trimethyl chitosan, Retinoic acid, Glioma, Anti-cancer, Cytotoxicity, Apoptosis*

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INTRODUCTION

Glioblastoma is a harmful and often fatal brain malignancy, with approximately 23, 000 cases diagnosed in adults in the US each year [1,2]. Glioblastoma is regarded as a Grade IV tumor by the World Health Organization (WHO) [3,4]. The blood–brain barrier (BBB) provides a uniquely privileged immune environment that remains impenetrable to many small and large molecule therapeutics used for treatment [5].

Lipid-based carriers are within the submicron range in size (i.e., 100–300 nm); therefore, we developed a lipid-based carrier to deliver molecular therapeutics across the BBB to tumors [6,7]. Additionally, solid lipid nanoparticles (SLNs) have a solid lipid core that can effectively incorporate hydrophobic drugs. Lipid nanoparticles are non-toxic and biocompatible, with a good loading capacity, resulting in increased solubility and stability of the loaded drugs; sustained delivery of hydrophobic drugs using lipid nanoparticles has been reported [8,9]. We designed surface-modified SLNs, which have

been used as a system to successfully deliver drugs across the BBB to tumor tissues with high specificity [10].

Chitosan (CS), a cationic natural polymer, exhibits excellent biomedical properties for the sustained release of therapeutic agents; it is biodegradable, biocompatible, modifiable, and non-toxic [11]. Chitosan, N-deacetylated chitin, can be derivatized by N-methylation to form N, N, N-trimethyl chitosan (TMC). CS has several moieties that can readily be methylated to change its form from a trimethyl group into a polysaccharide backbone, which improves its aqueous solubility over all pH ranges because of its polyelectrolytic cationic nature, whereas CS is most soluble in acidic environments at pH < 6 [12]. TMC also possesses mucoadhesive and absorption-enhancing properties because it can transiently open tight junctions between epithelial cells without cytotoxicity [13]. Thus, TMC has potential in targeted drug-delivery applications.

Retinoic acid (RA), a derivative of vitamin A, is a potential anti-cancer agent [14]. RA action is mediated by binding of RA receptors present in the nucleus of carcinoma cells, which then bind to regulatory regions of specific target genes, followed by growth inhibition and apoptosis in brain carcinoma cells. However, the poor aqueous solubility of RA is a major hurdle for its intravenous (i.v.) administration; therefore, RA incorporated into surface-modified lipid-based carriers could be a promising means to overcome its solubility limitations [15,16].

In this study, we developed stable RA-loaded SLNs (R-SLNs) and subsequently investigated their surface modification with TMC (TR-SLNs). Furthermore, we performed several physicochemical characterizations, morphological imaging, and assessed the *in vitro* release patterns of R-SLNs and TR-SLNs. Cytotoxicity and annexin V/propidium iodide (PI) staining studies were performed in U87MG cells that showed improved anti-cancer efficacy in delivering RA across the BBB to inhibit brain tumor growth, indicating increased therapeutic bioavailability.

EXPERIMENTAL

Materials

Compritol® 888 ATO and Tween 80 were from BASF (Ludwigshafen, Germany). Retinoic acid was purchased from TCI (Korea). Cholesterol and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (UK). An annexin V/PI staining apoptosis kit was obtained from BD Biosciences (UK). U87MG cells were purchased from the American Type Culture Collection (ATCC; USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Beijing, China), supplemented with 10 % fetal bovine serum (FBS; Hyclone) in a 5 % CO_2 atmosphere at 37 °C.

Preparation of TMC

TMC was synthesized by reductive methylation according to a previous report with some modifications [17]. Briefly, 2 g chitosan, 1.3 g sodium azide, 4 mL sodium hydroxide (10 % w/v), and 3.5 mL methyl iodide were added and stirred at 50 °C for 2 h. Next, 150 mL of distilled water (DW) was added and stirring was continued for 1 h before being dialyzed against DW (3, 500 Da cut-off) overnight. A precipitate was formed by adding a large volume of acetone; it was washed with sodium hydroxide. The precipitate was suspended in water and dialyzed for 24 h, followed by freeze drying.

Preparation of nanoparticles

SLNs were prepared by a homogenization method followed by probe sonication (Figure 1). The lipid phase, consisting of Compritol® 888 (250 mg), stearic acid (50 mg), and cholesterol (30 mg), was heated and melted. RA (10 mg) was then dispersed in the hot lipid phase, after which a preheated aqueous phase (250 mg), and later Tween 80, was added to the lipophilic phase and homogenized using an Ultra-Turrax® homogenizer (3 min, 13,000 rpm). The resulting pre-emulsion was then sonicated using a probe sonicator for 5 min. The synthesized TMC was added to the surface of the SLNs by adding at a ratio of 30: 1 (w/w) and stirred for 6 h. Finally, the reaction was centrifuged (11, 000 rpm, 15 min). The resulting TMC-SLNs were lyophilized and stored at 4 °C.

Dynamic light scattering (DLS)

The mean particle size, polydispersity index (PDI), and zeta potential (ZP) of the TR-SLNs were measured using an ELSZ-1000 (Photal Otsuka Electronics, Japan), based on the DLS method. Data are expressed as means ± standard deviation (SD).

Morphological characterization

Field emission scanning electron microscopy (FE-SEM) of TR-SLNs was performed (JSM electron microscope; JEOL, Japan).

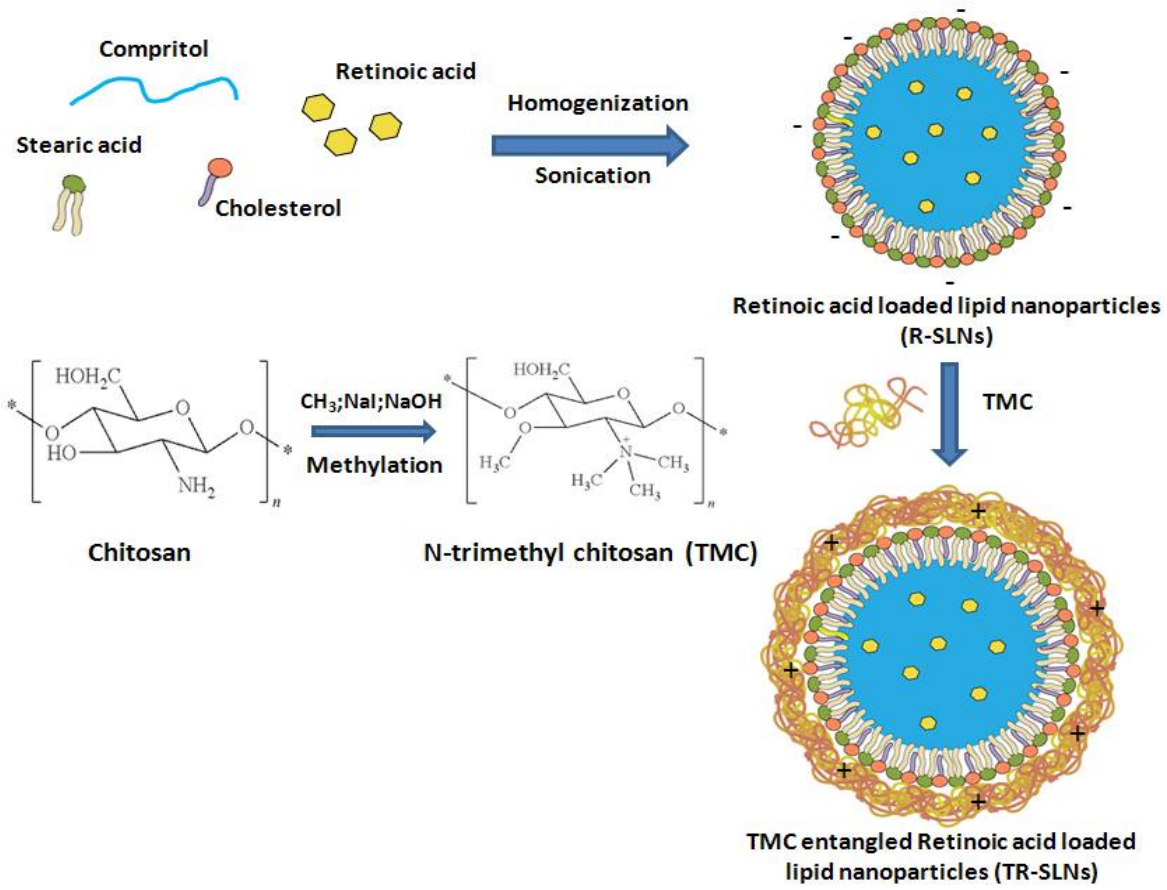

Figure 1: Schematic representation of the preparation of TMC-entangled RA-loaded solid lipid nanoparticles (TR-SLNs)

The freeze-dried samples were spread on carbon tape over a stub and vacuum-dried. Then, a gold coating was applied using an ion-sputtering device. The gold-coated samples were vacuum-dried and examined under the electron microscope.

Determination of encapsulation efficiency (EE)

EE was estimated by high-performance liquid chromatography (HPLC). Briefly, 1 mL of the formulation was centrifuged (5, 000 rpm, 30 min, 25 °C). The TMC-coated RA-loaded SLNs were then dissolved in tetrahydrofuran and diluted in the mobile phase. The mobile phase consisted of acetonitrile, DW, and phosphoric acid (75: 20: 0.1) at a flow rate of 1 mL/min. This dispersion was filtered through in a 0.45-µM membrane filter (Millipore, US) and analyzed by HPLC at a wavelength of 340 nm. The encapsulation efficiency (E %) was calculated using Equation 1:

$$E\ (\%) = \{(R_t - R_f)/R_t\} \times 100$$

where R_t and R_f are the total amount of RA and amount of free RA, respectively.

In vitro drug release studies

The *in vitro* release study was performed using a dialysis method. RT-SLNs (1 mL) were placed in a dialysis bag (MW cut-off, 10 kDa), which was then suspended in PBS buffer, pH 7.4, as the release medium. Samples were kept in a shaking water bath at 37 °C with gentle stirring (100 rpm). At stipulated time intervals, the buffer (10 mL) was replaced with fresh medium to maintain sink conditions, and the % drug released was analyzed using the HPLC-UV system described above. Data are expressed as means ± SD.

Cytotoxicity assay

Briefly, 1.5×10^4 U87MG cells were seeded in a 96-well plate in 100 µL of medium and incubated for 24 h. Then, they were treated with various formulations (RA, R-SLNs, and TR-SLNs) at increasing concentrations, ranging from 0.01 to 25 µg/mL, and with blank carrier SLNs and T-SLNs (0.001 to 500 µg/mL). The formulations were incubated for 24 h. Then, cells were washed with PBS and treated with MTT solution (5 mg/mL) and incubated for a further 4 h. The resulting formazan crystals were then dissolved

in 0.1 mL of DMSO. The plate with the mixture was gently shaken before measuring the absorbance at 570 nm on a microplate reader. Each experiment was repeated six times (n = 6).

Apoptosis assay

U87MG cells were seeded at a density of 2 × 10^5 cells/well in a 6-well plate and incubated for 24 h. The cells were treated with free RA, R-SLNs, and TR-SLNs for 24 h. Then, the cells were trypsinized, washed, collected in PBS, and stained with annexin-V and PI for 15 min. Finally, the cells were assessed using a FACSCalibur™ flow cytometer (shanghai, China).

Statistical analysis

Student's *t*-test was used to compare differences between groups. P values < 0.05 were considered to indicate statistical significance. Statistical evaluations were performed using the Mann–Whitney U test. SPSS software (ver. 20.0; SPSS Inc., IL, USA) was used. Experiments were performed at least in triplicate and data are presented as means ± SD.

RESULTS

Characteristics of R-SLNs and TR-SLNs

The mean particle size was 148 ± 2.41, the polydispersity (PDI) was 0.124, and the ZP was −21.2 ± 1.47. After the TMC coating was applied to the surface of SLNs, the particle size was increased to 214 ± 3.45 nm and the PDI was 0.214 ± 0.01, with a marked increase in charge due to the strong positive nature of TMC compared with bare SLNs (Figure 2).

RA caused a marked increase in the viscosity, resulting in an increased nanoparticle size. These results confirmed the presence of TMC on the surface of the nanocarriers. Lipid nanocarriers have recently gained attention for improving the delivery of hydrophobic drugs due to the high affinity of these small molecules for the lipid core. The EE of retinoic acid was high (86.4 ± 0.16 % for R-SLNs; 92.3 ± 0.08 % for TR-SLNs) with increased cholesterol amounts due to extra voids and rigidity between lipid molecules, thus reducing the 'leakage' of the small molecule from the solid lipid core of the SLNs. Figure 3 shows SEM images of TR-SLNs: they appeared to be smooth and spherical with a uniformly distributed size, indicating that the shape of the nanoparticles was maintained after freeze-drying.

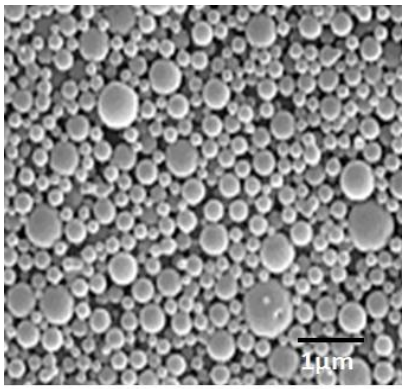

Figure 3: SEM image of TR-SLNs

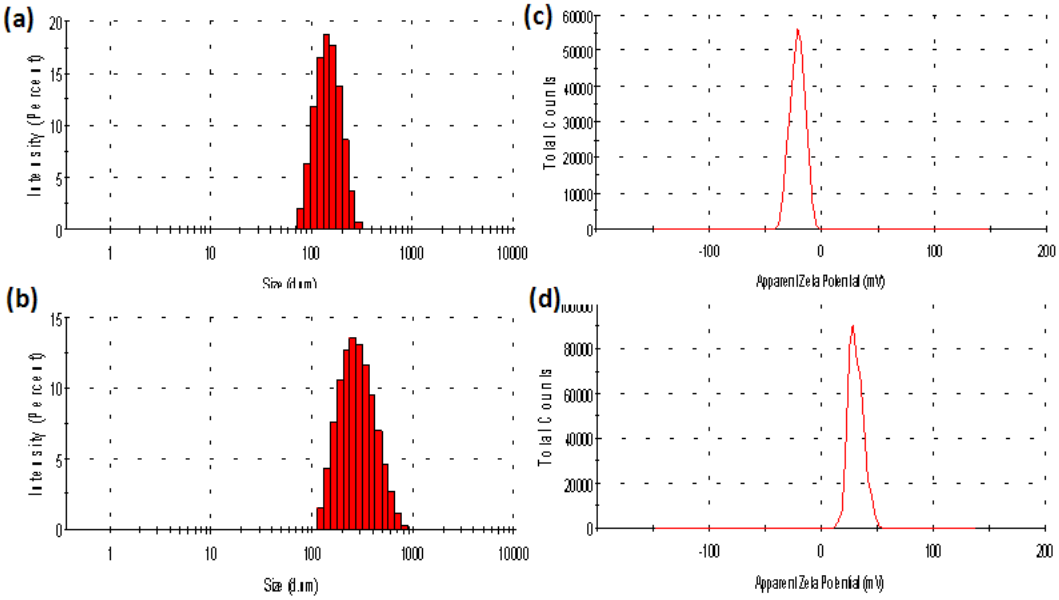

Figure 2: DLS characteristic images of (a) R-SLNs and TR-SLNs (PS) and (b) ZP

In vitro release

As shown in Figure 4, R-SLNs and TR-SLNs exhibited interesting release behaviors. Initially, R-SLNs showed a biphasic release pattern with an initial burst release over the first 10 h, with almost 42.8 ± 3.74 % of the drug being released, followed by sustained release for over 2 d, with approximately 58.2 ± 2.58 % of the drug being released. The TR-SLNs showed a monophasic release behavior throughout the study period, with 38.4 ± 1.14 % of the drug being released.

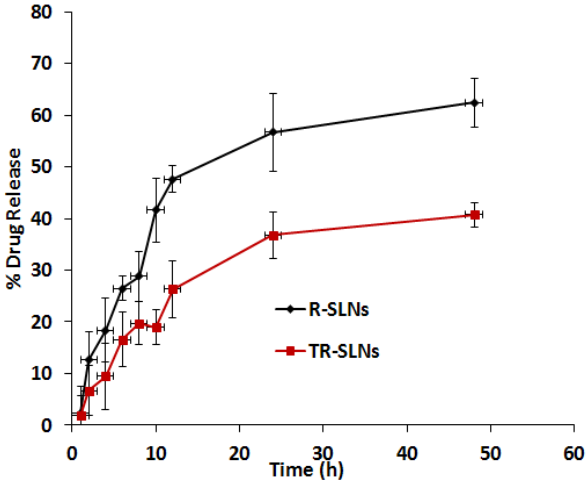

Figure 4: Drug release of RA from R-SLNs and TR-SLNs in PBS. The experiment was performed in triplicate (n = 3)

The initial burst release suggests the presence of small molecules adsorbed on the surface of the lipid nanoparticles, whereas the slower release suggests small molecules diffusing from the inner core of the lipid nanoparticles. The presence of TMC on the surface of the nanoparticle reflects the reduced, slower, and sustained release of the drug from the lipid core.

Cell viability

The *in vitro* effects of RA, R-SLNs, TR-SLNs, and blank carriers on cell viability were investigated in U87MG cells 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide using the MTT assay. As seen in Figure 5, cell viabilities with unloaded SLN and T-SLNs were very high, with 85 % viability even at 24-h incubation when tested with various concentrations.

Figure 6 shows that free RA was strongly cytotoxic at 10 µg/mL, with cell viability reduced to 62.2 ± 2.41 % after 24 h. The formulations exhibited concentration-dependent toxicity at different concentrations (0.01, 0.1, 1, 10, and 25 µg/mL) throughout the study period.

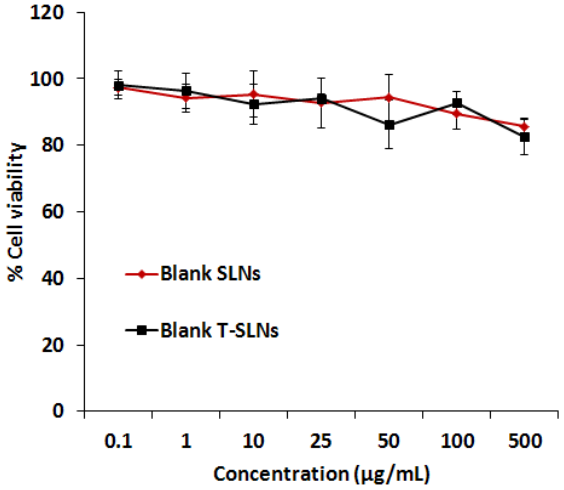

Figure 5: Cytotoxicity of blank SLNs and T-SLNs at different concentrations (*n* = 6)

The surface-modified TR-SLNs exerted an *in vitro* anti-tumor inhibitory effect of 48.2 ± 2.84 % at 10 µg/mL and 32.4 ± 3.5 % at 25 µg/mL, which was higher than those observed with free drug and R-SLNs at all tested concentrations ($p < 0.01$). The increased cytotoxicity with time suggests that the amount of drug released from the lipid core would increase with the prolonged circulation of TR-SLNs.

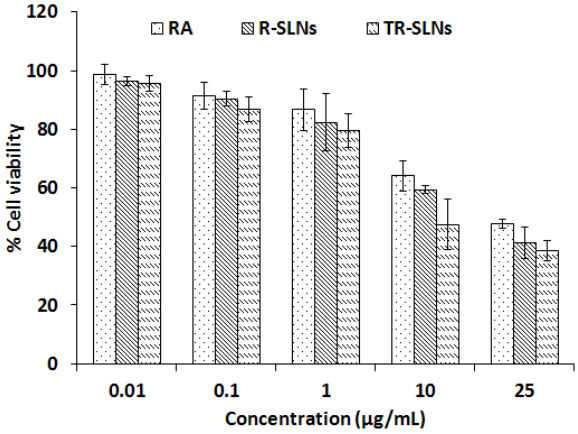

Figure 6. Cytotoxicity assay of free-drug RA, R-SLNs, and TR-SLNs at different concentrations (*n* = 6)

These results are similar to those of the blank carrier, indicating a negligible amount of cytotoxicity. The differences in cytotoxicity induced by the free drug and final formulations were significant.

Annexin V/PI staining

As shown in Figure 7, the apoptosis induced by RA, R-SLNs, and TR-SLNs was analyzed by flow cytometry.

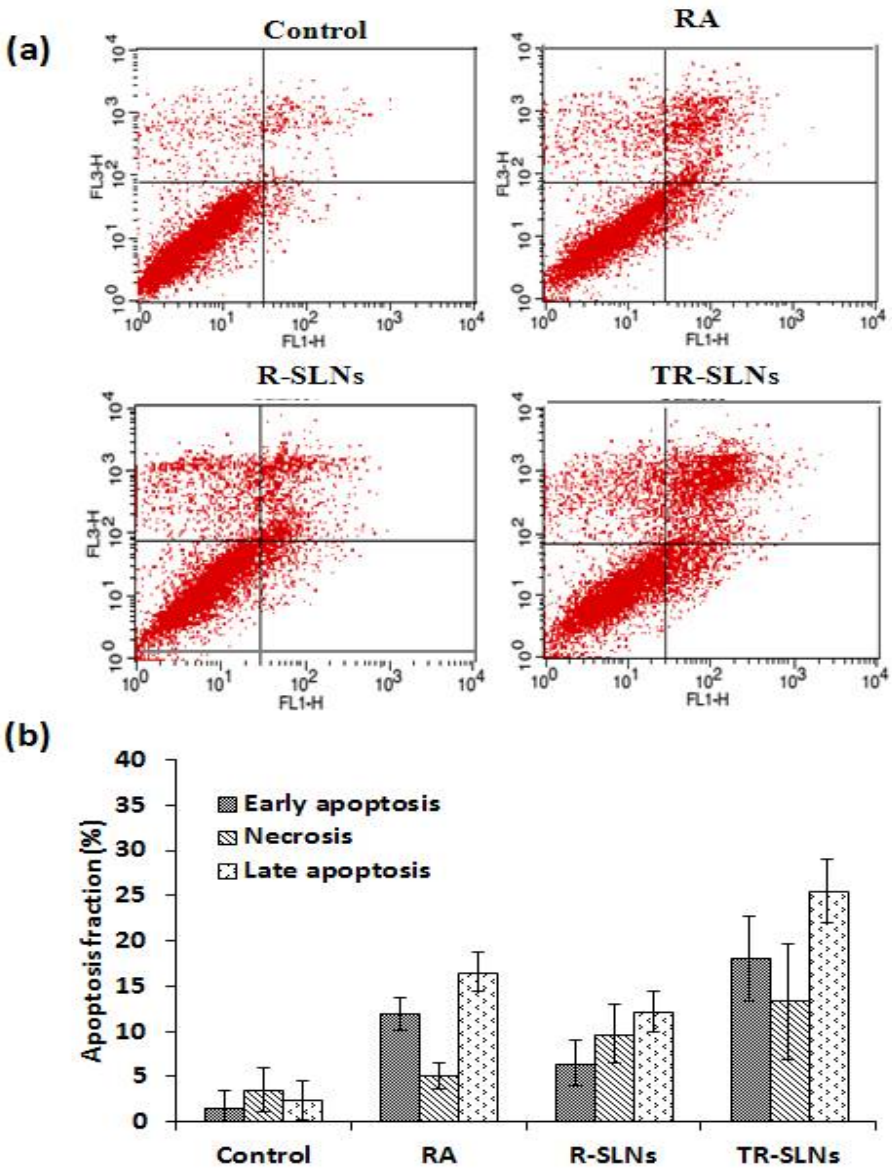

Figure 7: (a) Qualitative and (b) quantitative analysis of annexin V/PI staining of free RA, R-SLNs, and TR-SLNs in U87MG cells by flow cytometry

Of the cells treated with free RA (10 µg/mL), 11.91 ± 1.47 % were in early apoptosis, while 16.57 ± 2.4 % were in late apoptosis/early necrosis. Similarly, when treated with R-SLNs, 6.5 ± 2.8 % of cells were in early apoptosis and 12.26 ± 0.21 % were in late apoptosis. Interestingly, when the cells were treated with TR-SLNs, the cell populations in all phases were increased markedly, with 18.07 ± 0.1 %, 25.44 ± 1.2 %, and 13.35 ± 2.7 % in the early and late apoptotic, and necrotic phases, respectively, compared with the control group.

These results suggest that free RA, R-SLNs, and TR-SLNs also induced apoptosis at 10 µg/mL with a 24-h incubation time. These data are consistent with the cytotoxic data confirming the enhanced toxicity of the formulation in treating glioblastoma cells.

DISCUSSION

Glioblastoma is one of the most aggressive, prevalent, and fatal brain malignancies, with over 8,000 cases diagnosed in the US per year [18]. RA-loaded SLNs represent an alternative treatment for this brain cancer. RA is a metabolite of vitamin A and has many physiological effects in cancer and skin diseases [19]. However, due to its poor solubility in the aqueous phase, a drug delivery system is needed; in this study, we encapsulated RA in SLNs. SLNs have gained attention as a particulate system to improve the delivery and stability of drugs [20,21].

RA-loaded SLNs were prepared by high-pressure homogenization with a mean particle

size of 148 ± 2.41 nm, a PDI of 0.124, and a ZP of −21.2 ± 1.47. The size of the particle was increased versus bare SLNs after coating with TMC on the surface: 214 ± 3.45 nm and a PDI of 0.214 ± 0.01, with a marked increase in charge due to the strongly positive nature of TMC. According to a previous study [22], the amount of surfactant added in SLN dispersions is important in enhancing the drug-loading efficiency. The initial burst release is due to some of the drug molecules being adsorbed on the surface of the SLN particles, whereas the prolonged circulation pattern of TR-SLNs indicates drug diffusion from the inner matrix of the lipid core (~38 %).

Cytotoxic effects in U87MG cells were investigated using the MTT assay. Increased cytotoxicity of RA-loaded SLNs was observed compared with free RA. The results demonstrated that the cationic polymer-functionalized lipid nanocarrier was more cytotoxic than R-SLNs, resulting in concentration-dependent toxicity. Increased cytotoxicity in conjunction with increased concentration indicates an increased amount of drug released from the lipid matrix, suggesting a sustained release effect of TR-SLNs. These results indicated that free RA was not significantly more cytotoxic than R-SLN, and the blank SLNs exhibited no significant effect on cell viability. Furthermore, apoptosis induction was detected by annexin V/PI staining with flow cytometry. After the cells were treated with TR-SLNs, significant increases in early and late necrosis and apoptosis were observed when compared with the control. These data suggest greater activity of SLNs, in which RA is loaded with the lipid matrix of the SLNs, enabling improved drug uptake by tumor cells.

CONCLUSION

A novel nanocarrier system, RA-loaded surface-modified TMC-coated SLNs has been developed using a homogenization and sonication method. The spherical, uniformly distributed nano-sized particles (TR-SLNs) demonstrate sustained release behavior. TMC provides excellent protection to minimize the initial burst release and acts as a suitable carrier to improve the bioavailability of RA. The findings also show that the developed product exhbits anti-cancer activity. Thus, the optimized TR-SLNs are a potential drug delivery carrier for the treatment of brain cancer.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

REFERENCES

1. *Wen PY, Kesari S. Malignant gliomas in adults. N Engl J Med. 2008; 359: 492–507.*
2. *Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW, Kleihues P. The 2007 WHO classification of tumours of the central nervous system. ActaNeuropathol. 2007; 114: 97-109.*
3. *Croteau D, Mikkelsen T. Adults with newly diagnosed high-grade gliomas. Curr Treat Options Oncol. 2001; 2: 507–515.*
4. *Phuong LK, Allen C, Peng KW, Giannini C, Greiner S, TenEyck CJ, Mishra PK, Macura SI, Russell SJ, Galanis EC. Use of a vaccine strain of measles virus genetically engineered to produce carcinoembryonic antigen as a novel therapeutic agent against glioblastoma multiforme. Cancer Res. 2003; 63: 2462–2469.*
5. *Rajadhyaksha M, Boyden T, Liras J, El-Kattan A, and Brodfuehrer J. Current advances in delivery of*

biotherapeutics across the blood-brain barrier. Curr. Drug Discovery Technol. 2011; 8: 87−101.

6. *Abbott NJ, Romero IA. Transporting therapeutics across the blood–brain barrier. Mol. Med. Today. 1996; 2; 106–113.*
7. *Aryal M, Arvanitis CD, Alexander PM, McDannold N. Ultrasound-mediated blood–brain barrier disruption for targeted drug delivery in the central nervous system. Adv. Drug Deliv. Rev. 2014; 72: 94–109.*
8. *Lim S, Kim C. Formulation parameters determining the physicochemical characteristics of solid lipid nanoparticles loaded with all-trans retinoic acid. Int. J. Pharm. 2002; 243: 135–146.*
9. *Hu L, Tang X, Cui F. Solid lipid nanoparticles (SLNs) to improve oral bioavailability of poorly soluble drugs. J. Pharm. Pharmacol. 2004; 56: 1527–1535.*
10. *Garanti T, Stasik A, Burrow AJ, Alhnan MA, Wan KW. Anti-glioma activity and the mechanism of cellular uptake of Asiatic acid-loaded solid lipid nanoparticles. Int J Pharm. 2016; 500: 305-315.*
11. *Agnihotri SA, Mallikarjuna NN, Aminabhavi TM. Recent advances on chitosan-based micro- and nanoparticles in drug delivery. J Control Release. 2004; 100: 5–28.*
12. *Chen F, Zhang Z-R, Yuan F, Qin X, Wang M, Huang Y. In vitro and in vivo study of N-trimethyl chitosan nanoparticles for oral protein delivery. Int J Pharm. 2008; 349: 226–233.*
13. *Subbiah R, Ramalingam P, Ramasundaram S, Kim DY, Park K, Ramasamy MK, et al. N, N, N-Trimethyl chitosan nanoparticles for controlled intranasal delivery of HBV surface antigen. Carbohydr Polym. 2012; 89: 1289–1297.*
14. *Tang XH, Gudas LJ. Retinoids, retinoic acid receptors, and cancer. Annu Rev Pathol. 2011; 6: 345–364.*
15. *Bushue N, Wan YJ. Retinoid pathway and cancer therapeutics. Adv Drug Deliv Rev. 2010; 62: 1285–1298.*
16. *Fang J, Chen SJ, Tong JH, Wang ZG. Treatment of acute promyelocytic Leukemia with ATRA and As2O3: a model of molecular. Cancer Biol Ther 2002; 1: 614–620.*
17. *Polnok A, Borchard G, Verhoef JC, Sarisuta N, Junginger HE. Influence of methylation process on the degree of quaternization of N-trimethylchitosan chloride. Eur J Pharm Biopharm. 2004; 57: 77-83.*
18. *Grossman SA, Ye X, Piantadosi S, Desideri S, Nabors LB, Rosenfeld M, Fisher J. Survival of patients with newly diagnosed glioblastoma treated with radiation and temozolomide in research studies in the United States. Cancer Res. 2010; 16: 2443–2449.*
19. *Lucek RW, Colburn WA. Clinical pharmacokinetics of the retinoids. Clin Pharmacokinet. 1985; 10: 38–62.*
20. *Ruttala HB, Ramasamy T, Poudal BK, Choi Y, Choi JY, Kim J, Ku SK, Choi HG, Yong CS, Kim JO. Molecularly targeted co-delivery of a histone deacetylase inhibitor and paclitaxel by lipid-protein hybrid nanoparticles for synergistic combinational chemotherapy. Oncotarget. 2017; 8: 14925-14940.*
21. *Ruttala HB, Ramasamy T, Shin BS, Choi HG, Yong CS, Kim JO. Layer-by-Layer Assembly of Hierarchical Nanoarchitectures to Enhance the Systemic Performance of Nanoparticle Albumin-bound Paclitaxel Int. J. Pharm 2017; 519: 11-21.*
22. *Tiyaboonchai W, Tungpradit W, Plianbangchang P. Formulation and characterization of curcuminoids loaded solid lipid nanoparticles. Int. J. Pharm.2007; 337: 299–306.*