DoE Directed Optimization, Development and Characterization of Resveratrol Loaded Nlc System for the Nose to Brain Delivery in the Management of Glioblastoma Multiforme

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Research Article

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Abstract

Nose to brain delivery of resveratrol can be a very useful method to overcome the limitations possessed by conventional delivery approaches namely, hepatic metabolism, low bioavailability and half-life of resveratrol, and presence of blood-brain barrier (BBB). The objective of this research work was to develop and optimize the resveratrol-loaded NLCs and coating these carriers with chitosan to increase the residence time of the formulation into the nasal cavity and enhanced permeation across the nasal mucosa. Three CQAs (Particle size, Entrapment efficiency, and PDI), and CMAs (Solid: total lipid concentration, surfactant concentration, and bioactive amount) were selected and the formulation was optimized using the Box-Behnken design (BBD) approach. The optimized batch was evaluated for physicochemical characteristics such as particle size (168.24 ± 8.24 nm), PDI (0.151 ± 0.003), and entrapment efficiency (77.42 ± 3.76 %). This optimized batch was coated with chitosan, which produced coated NLCs with a particle size of 317.7 ± 15.9 nm, and PDI was 0.089 ± 0.009 . The morphological study using TEM confirmed the spherical shape, size, and surface coating of the NLCs. Furthermore, both the uncoated and coated particles were analyzed for *in vitro* resveratrol release, *ex vivo* diffusion study, and antioxidant assay. NLCs was founded to show sustained *in vitro* release characteristic, and enhanced bioactive diffusion across the nasal mucosa compared to the bioactive solution of resveratrol. The antioxidant assay revealed that the antioxidant property of resveratrol was intact in the formulation, and a slight increase in antioxidant activity of the formulation was also observed which may be due to the presence of sesame oil in the formulation. These results indicated that the chitosan-coated NLCs can be used to deliver therapeutic moieties more efficiently via the nose to brain drug delivery.

1. Introduction

Glioblastoma multiforme (GBM) is the most common, and aggressive brain tumor with an average prevalence rate of 3 per 100000 people [1–3], and a very low average survival rate (15 months post-diagnosis) [4, 5]. WHO has further classified GBM into three types based on the different genetic mutations. Among all, the most prominent and aggressive class is De-novo or IDH-wild type glioblastoma which comprises approximately 90 % of cases of GBM. The others are IDH-mutant (IDH-I and IDH-II) and Nitric oxide synthase (NOS) and collectively cover the remaining 10 % cases of the GBM [6, 7]. Currently, there is no curative treatment available for GBM. The standard practice for the management of GBM is the surgical elimination of the tumor followed by the treatment with radiotherapy and chemotherapy [2]. The post-treatment patient survival rate after this therapy is very poor, which makes GBM the most deadly brain tumor. Out of the several reasons responsible for the untreatable nature of GBM, the two main reasons are the development of chemoresistance in the tumor against the existing chemotherapeutics because of genetic mutations, and the tumor reoccurrence after the treatment. Additionally, the blood-brain barrier (BBB) limits the permeation of chemotherapeutics into the brain because of structural features such as tight junctions, absence of fenestration, and presence of p-glycoprotein efflux system [8]. To overcome these hurdles in the treatment of GBM, the increased chemotherapeutic dose is administered to the patient for providing the effective therapeutic concentration inside the tumor, which

further results in increased side effects and compromised quality of patient life. These treatment obstacles and toxicity of the current therapy indicate the need for an effective and non-toxic therapeutic moiety against GBM. Resveratrol is a natural phytoalexin, stilbenes compound commonly found in red wine, grapes, and Soybeans. Stilbenes are the secondary metabolites of the plants produced in response to stress factors such as fungal infections, and UV radiations. Resveratrol is a photosensitive compound and exists in two isomeric forms, *cis*-resveratrol and *trans*-resveratrol. Among these, the *trans*-resveratrol is the most investigated isomer for various therapeutic outcomes such as anti-oxidant, anti-inflammatory, anti-cancer, cardio-protective, and neuroprotective activity. Resveratrol is a proven anti-cancer molecule that acts on the multiple targets involved in all four stages of tumor namely, initiation, promotion, and progression [9–11]. Resveratrol shows anti-carcinogenic activity by suppressing the abnormal signaling inside the cell, reducing the genetic alteration due to oxidative stress, shrinking the abnormal neoangiogenesis, and inhibiting the increased production of growth hormone in the tumorous tissue. Moreover, the anti-inflammatory, immunomodulatory, and anti-oxidant effects further enhance the anti-cancer activity of resveratrol [12]. Despite the multiple molecular targets and significant efficacy against glioma, the poor solubility, bioavailability, and high metabolism collectively decrease the potential anticancer activity of resveratrol. Upon administration, resveratrol metabolizes into glucuronide and sulfate conjugates, decreasing the oral bioavailability to only 9–14 minutes. Furthermore, the photosensitivity and oxidation of resveratrol also reduce the chemical stability and pharmacological activity of the bioactive [11, 13, 14]. To overcome these limitations of oral and/or parenteral administration of resveratrol, scientists are exploring nose to brain (NTB) drug delivery as an alternative administration route for the CNS disorders such as Parkinson's, Alzheimer's, and GBM [15–17]. Drug molecules can be delivered non-invasively into the brain via the single or combination of several pathways such as olfactory, trigeminal nerves, cerebrospinal fluid, and lymphatic pathways, bypassing BBB, avoiding first-pass metabolism, and circumventing the systemic absorption of the drug [18]. Despite the mentioned supremacy, the NTB route also possesses one or more loopholes such as, low permeation across the nasal epithelium, enzymatic degradation of drug molecules, very low administration volume, and mucociliary clearance [19].

To outweigh these limitations, lipidic nanocarrier systems such as nanostructured lipidic carrier (NLCs), can be deployed in drug delivery via the NTB route because of their number of inherent features, for instance, increased permeation and bioavailability of the drug, protection of drug molecule from chemical, and enzymatic degradation, and controlled drug release [20]. Additionally, owing to their smaller size, these can accumulate into the tumorous tissue by enhanced permeation and retention effect (EPR), which is responsible for passive targeting of the drug inside the tumor tissue [21]. Moreover, the easily modifiable surface of these nanoparticles also provides the scope for active targeting of the tumor, which can be achieved by the surface conjugation with the targeting moieties such as, a fragment of an antibody, folic acid, albumin, and transferrin, particularly for GBM [20]. The receptors of mentioned ligands are highly proliferated inside the tumor because of the increased demand for nutrients such as iron, and amino acids, required for the rapid proliferation of the tumorous tissue. After administration, these fabricated nanocarriers rapidly diffuse into the brain. Where, they are actively uptaken by the

respective receptors inside the tumor microenvironment, hence releasing more of the drug load inside the targeted cancerous tissues, and minimizing the side effects of the drug to the normal brain tissues [22, 23]. In this work, the NLC system is optimized by the FbD (Formulation by Design) approach [24, 25]. An experimental design is a structured, organized method for determining the relationships between factors affecting a process and the output of that process. It is also known as “Design of Experiments” (DoE). In other words, the latter is the means of achieving process knowledge, through the establishment of mathematical relationships between process inputs and their outputs [26, 27]. Thus in the present work, we hypothesized that the resveratrol is a proven anticancer molecule due to its abilities to modulate pro-inflammatory cytokines, mitochondrial dysfunction, and oxidative stress, and the nasal route is highly effective as compared to the oral/iv route as direct localized action on the brain, rapid onset of action, no hepatic first-pass metabolism and high bioavailability. So, the development of chitosan-coated NLCs of Resveratrol for the nose to brain delivery with enhanced therapeutic efficacy, increased bioavailability, and brain distribution of resveratrol in the brain is envisaged.

2. Materials And Methods

2.1 Materials

Resveratrol was obtained as a gift sample from the MMG Healthcare Ltd., Sirmour, Himachal Pradesh, Glycerol monostearate was purchased from CDH Pvt. Ltd., Delhi, Tween 80 was procured from SD Fine chemical Ltd., Mumbai, Dialysis membrane, and Pluronic® F68 were purchased from HiMedia Pvt. Ltd., Mumbai, Chitosan was acquired from Sigma-Aldrich, USA, DPPH was purchased from SRL Pvt. Ltd., Mumbai. All other reagents and chemicals used during the research were of analytical grade.

2.2 Methods

2.2.1 Screening of excipients i.e. solid lipid, liquid lipid, and surfactants

The selection of components was done based on a literature survey, availability, and prior experience. The Glycerol monostearate (GMS) was found to be the most common solid lipid used in the preparation of NLC systems, specifically for the nose to brain drug delivery. GMS is used widely in cosmetic, and food preparations worldwide. Hence, it was selected as a solid lipid for the resveratrol-loaded NLCs [28]. The sesame oil was selected as a liquid lipid because the high solubility of resveratrol is reported in this oil [29,30]. Sesame oil is also reported to have antioxidant and anti-inflammatory activities. In the pre-optimization studies, both solid and liquid lipids were found to be compatible with each other. Two surfactants i.e. Tween 80, and Pluronic®F68 were also selected based on the literature survey and preliminary studies [31,32].

2.2.2 Formulation of Resveratrol Loaded NLC by the hot-emulsification homogenization method

The NLCs were formulated by the Hot-emulsification homogenization method as described in figure 1. The solid lipid (GMS) and liquid lipid (sesame oil) were mixed in a fixed ratio. This mixture was heated at

a temperature above their melting point (80 ± 5 °C) to melt the solid lipid. The bioactive (resveratrol) was also added to the melted lipid mixture. The aqueous phase consisting of the dissolved surfactant (Tween 80), and stabilizer (Pluronic F68®) was also heated at the same temperature. After heating, both lipidic and aqueous phases were mixed and homogenized (IKA® T25 digital ultra turrax, Cole Parmer) at 10,000 RPM for 30 min. to form homogeneous micro-emulsion. This micro-emulsion was cooled down at 4 °C to form NLCs by solidification of solid lipid [24].

2.2.3 DoE based optimization by applying Box Behnken Design

Based on the pre-formulation studies, prior knowledge, and literature survey, three critical material attributes (CMAs) namely solid lipid: total lipid ratio (X1), surfactant concentration (X2), and amount of bioactive (X3), and three critical quality attributes (CQAs) namely, particle size (Y1), entrapment efficiency (Y2), and PDI (Y3), were selected for the systematic optimization of the formulation using the response surface methodology (RSM). The effect of individual factors and the combination of two or more factors on the quality parameters were statistically evaluated employing the Box-Behnken Design (BBD). According to this design, a total of 17 batches of the formulation were prepared, and each factor was varied in three predefined levels i.e. low (-1), medium (0), and high (+1), and evaluated for the decided critical quality characteristics. The actual values of each level for all three CMAs and requirements for the CQAs are mentioned in table 1. The generated data were analyzed using the Design expert software® trial version 13. The single and interactive effects of critical material attributes (CMA) affecting CQAs were depicted by the polynomial equations. These equations showing the relationship between CMAs and CQAs were analyzed by fitting into different linear regression models and were further validated using the analysis of variance (ANOVA) test. This complex relationship between the CMAs and CQAs was further illustrated by the model plots such as contour plots, perturbation plots, and 3D surface plots. The desired values for each CQA were fed into the software, after which the batches with optimum CQAs were predicted by the software using the desirability approach, and checkpoint analysis [33–35].

According to the design, 17 formulations were prepared and evaluated for response. All the batches were evaluated for particle size, PDI, and entrapment efficiency, and the data were analyzed using the Design-Expert 13.0.2 (trial version), Stat-Ease, USA.

2.2.4 Development of chitosan-coated NLCs

For the preparation of chitosan-coated NLCs, chitosan solution (0.2 %) was prepared in 1 % v/v acetic acid solution. Chitosan solution (5 mL) was added dropwise into 10 mL of optimized formulation (1:2 ratio) under smooth magnetic stirring at 300 RPM for 15 min. at room temperature [36,37].

2.2.5 Morphology/Transmission Electron Microscopy

The morphology of the micro-emulsion was observed using a transmission electron microscope (TEM) attached with a mega view II digital camera (H 7500, Hitachi, Tokyo, Japan). A drop of sample diluted

with water was placed on a copper grid and the excess was drawn off with a filter paper. The image was magnified and focused on a layer of the photographic film [22].

2.2.6 Particle Size and Polydispersity Index Determination

The mean particle size and polydispersity index of the NLC dispersion were determined by light scattering based on laser diffraction using the particle size analyzer (Beckman Coulter Counter Delsa Nano C, USA, DLS 4 C) [38].

2.2.7 *In-Vitro* release study

In vitro release profile of Res. Loaded chitosan-coated NLCs, Res. Loaded NLCs and bioactive suspension were studied in phosphate buffer: methanol (pH=7.4) in 7:3 ratio using dialysis membrane. Dialysis membranes were kept in the distilled water overnight and then exposed to running water for 2 h to remove glycerin-based contents. These Dialysis membranes were treated with a strong alkaline solution (0.3% Sodium sulfide) heated up to 80°C for 1 min and then washed with hot water at 60 °C for 3 min. These dialysis membranes were further exposed with an acidic solution (0.2% H₂SO₄) for 2 min and washed with warm water to remove any excess acid content on the surface of the membrane. Treated membranes were kept in the dissolution media for 12 hours before the use. *In vitro* bioactive release studies were performed using USP dissolution test apparatus (Labindia, DS 8000, India) consist of 200 mL media vessel and enhancer cell at the rotation speed of 50 RPM in PBS: methanol (pH 7.4) in 7:3 ratio as release medium, and temperature at 32 ± 0.5 °C. Dialysis membranes with accurately measured quantities of bioactive suspension or chitosan-coated NLCs or uncoated NLC formulation (equivalent to 0.5 mg/mL resveratrol) were tightly packed into the enhancer cells. These enhancer cells were placed into the vessels containing dissolution media. The volume and temperature of the dissolution medium for each sample were 150 ml and 32 ± 0.5°C. At predetermined time intervals, samples (3 mL) were withdrawn, replaced with the same volume of new media, and assayed for bioactive content at λ_{max} 306 nm against a blank (dissolution media) using UV–Visible spectrophotometer. Mean results of triplicate measurements and standard deviation were reported. Based on various equations, the release kinetic model followed by the formulations was calculated [39].

2.2.8 *In vitro* DPPH assay for antioxidant activity

The antioxidant activity of the Res. loaded NLCs, blank NLCs is compared with the bioactive suspension using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay method. The DPPH are stable free radical molecules, which gave deep violet color in methanol and converts into pale yellow solution when neutralize by the presence of the antioxidant compound. The solution of DPPH (0.004 %) was prepared in methanol and kept in dark. The 2.5 mL of DPPH solution was mixed with 1.5 mL of Res. suspension (1mg/1.5mL), blank NLCs, Res. loaded NLCs, Ascorbic acid, and distilled water. These solutions were stored in dark for 30 min after which absorbance was measured of all the solutions at 517 nm using methanol as blank. The percentage of free radical inhibition activity was calculated using the following formula-

$$DPPH \text{ inhibition \%} = \left[\frac{A_0 - A_1}{A_0} \right] * 100$$

Where A_0 is the absorbance of blank (DPPH solution with distilled water), and A_1 is the absorbance of bioactive solution or formulations (Res. loaded or blank). All the experiments were performed in triplicate and the data were analyzed using software [40].

2.2.9 *Ex Vivo* permeation study

Drug permeability was done by the Franz cell diffusion (having 50 mL volume and 2.85 cm² surface area) method using the freshly excised nasal skin of the sheep. The skin was washed and equilibrated with the media for 1 h before the experimentation. The animal skin was fixed between the receptor and donor compartment, and the formulations and drug solution, each equivalent to 1 mg of resveratrol was placed into the donor compartment. The PBS: Methanol buffer (pH=7.4) was filled inside the receptor compartment and the study was conducted on 100±2 RPM stirring speed and at 32° ± 0.5 °C temperature. The 3 mL sample was withdrawn from the receptor compartment on each predetermined time interval (1,2,4,6,8,10,12,14,16,18,24 hours) and replaced with the same amount of fresh media. These samples were analyzed spectroscopically for the diffused amount of resveratrol on each time interval. The percentage cumulative amount of resveratrol diffused across the skin membrane was calculated and plotted against the time interval [35].

3. Results And Discussion

3.1 Optimization of Formulation

Optimization of Resveratrol-loaded NLCs was done by applying BBD to get desired particle size, polydispersity index, and maximum entrapment efficiency as shown in table 1. Quantitative aspects of the effects and relationships among various formulation variables of RES NLCs were investigated using Response Surface Methodology (RSM) using design-Expert software (trial version 13.0.2.0, Stat-Ease, Inc.). BBD Design with a total of 17 experimental runs was selected to optimize the various process parameters at three levels and evaluated for response.

3.1.1 Fitting the model to data

Table 2 shows the results of dependent variables, mean particle size (Y1), entrapment efficiency (Y2), and PDI (Y3) of the formulation. This generated data were fitted into different models using the design-expert software, and the best-fitted model was suggested by the software. The ANOVA technique was applied to validate the suggested model. The quadratic model was suggested by the software for all the CQAs, and when analyzed using ANOVA, this model was also found statistically significant ($P < 0.05$) for all the cases as depicted in table 3. The adjusted R^2 values and the predicted R^2 values for all the CQAs were in desired limits i.e. the difference between adjusted and predicted R^2 values were less than 0.2. Three polynomial equations were generated by the software for each CQA, specifying both the individual effect

and the interaction effect of all the CMAs on each CQA. Furthermore, the perturbation plots, 2D contour plots, and 3D response surface plots were generated by the software for illustrating the interaction effects of two CMAs on CQA at any particular time interval [41].

3.1.2 Influence of formulation variables on the response (CQAs)

Different factors amount solid lipid: total lipid (X1) and amount of surfactants (%) (X2), and amount of Res. (X3) affecting the different critical quality attributes of formulation i.e. particle size (Y1), entrapment efficiency (Y2), and PDI (Y3). The influence of variables on critical quality attributes is shown in figure 2 for particle size, for entrapment efficiency are shown in figure 3 and for PDI effects are shown in figure 4.

3.1.3 Response Y1 Particle size

The particle size of NLCs is a very critical quality factor of the formulation. The minimum particle size of the NLCs is required for successful bioactive delivery to the brain by permeating the nasal epithelium membrane. The nanocarriers having a particle size of less than 200 nm are found to accumulate into the brain via intracellular uptake pathways such as clathrin-dependent endocytosis, and caveolae-mediated endocytosis [38,42]. Similarly, nanocarriers less than 20 nm in size are reported to follow an extracellular transport mechanism to travel from nose to brain. Furthermore, nanocarriers with particle size range between 100-200 nm are reported to show enhanced permeation and retention effect (EPR) [43]. Due to the EPR effect, these nanoparticles may accumulate at the tumor site by extravasating through the leaky fenestrations present in tumor vasculature. Hence NLCs within 100-200 nm are more likely to exert higher concentration at the GBM site in the brain [44]. Based on the above information, particle size between 100-200 nm is desired for the maximum nose to brain transport of the bioactive.

$$\begin{aligned} \text{Particle size (Y1)} = & +259.90 + 60.00X_1 - 89.25X_2 + 24.35X_3 + 15.47X_1X_2 - \\ & 0.7250X_1X_3 - 29.63X_2X_3 + 15.81X_1^2 + 46.46X_2^2 + 14.01X_3^2 \end{aligned} \quad (\text{equation 1})$$

The polynomial equation, contour, and 3D response surface plot were indicating that the surfactant concentration (X2) has the highest impact upon the particle size followed by the solid lipid: total lipid ratio (X1), and amount of bioactive (X3). The amount of surfactant was found to have a negative relationship with the particle size. The higher amount of surfactant was found to give formulation with minimal size, which maybe because of the reduced surface tension between the two phases due to the long aliphatic side chain of Tween 80. Pluronic F-68 was also used in formulation, which acts as a stabilizer and co-emulsifier for the formulation by forming a layer on the interfacial surface and providing steric stabilization. Hence the combined use of both the surfactant possesses a strong effect on the particle size of the formulation. Moreover, the solid: total lipid ratio (X1), and bioactive concentration (X3) were found to exert a positive effect upon particle size. The particle size of the formulation was surging with the increased concentration of solid lipid, which may be because of the increased viscosity of the lipidic phase in the formulation. The high bioactive content also resulted in a larger particle size of the formulation.

3.1.4 Response Y2 Entrapment efficiency

Considering the small volume of the nasal cavity, entrapment efficiency and bioactive loading are other important formulation factors. The higher entrapment efficiency and bioactive loading are desirable to administer the required therapeutic amount of bioactive through a minimal volume of the formulation [45,46]. Good entrapment efficiency may be expected to promote the higher nose to brain bioactive uptake and reduce the frequency of administration for the formulation.

$$\left(\frac{1}{\text{Entrapment}(Y2)}\right) = +0.0241 + 0.0034X1 + 0.0034X2 - 0.0116X3 + 0.0033X1X2 - 0.0027X1X3 - 0.0019X2X3 - 0.0048X1^2 - 0.0013X2^2 + 0.0038X3^2 \quad (\text{equation 2})$$

After the data analysis by the software, the Box-Cox diagnostic plot suggested the inverse power transformation of data. From the generated polynomial equation, contour, and 3D response graphs plotted by the software, the positive highest effect on the entrapment efficiency was shown by the bioactive concentration (X3). The high resveratrol solubility in both solid, and liquid lipids may be the reason for this surge in entrapment efficiency. Surprisingly, Both the solid: total lipid (X1) ratio and the surfactant concentration (X2) were found to have an equivalent effect on the entrapment efficiency (Y2). The entrapment efficiency was found to be decreasing by increasing the amount of solid lipid. This may be indicating the high solubility of the bioactive in liquid lipid compared to the solid lipid. The amount of surfactant was also affected entrapment efficiency negatively because the increased concentration of surfactant in the formulation may solubilize more bioactive into the aqueous medium. Hence, letting out less amount of bioactive for encapsulation in the lipidic carrier.

3.1.5 Response Y3 PDI

The polydispersity index (PDI) is also another important formulation aspect particularly in the case of nanocarriers such as NLCs. It indicates the particle size distribution of the formulation [47]. The narrow particle size distribution is desirable for the better stability of the formulation. Ideally, a completely monodisperse formulation should have PDI equals to 0, and the range of PDI generally varies between 0.1 to 1 (48).

$$\frac{1}{\text{PDI}(Y3)} = +4.68 - 0.8698X1 + 1.26X2 - 0.1509X3 - 0.6934X1X2 + 0.0977X1X3 + 0.1733X2X3 - 0.1949X1^2 - 0.2487X2^2 - 0.4982X3^2 \quad (\text{equation 3})$$

The generated 3D response plots and polynomial equation were depicted that among all the CMAs, the PDI was most influenced by the surfactant concentration. The PDI was found to be decreasing by increasing the surfactant concentration. These results can be correlated with the surfactant concentration effect on particle size depicted in the previous section. The high surfactant concentration may reduce the particle size and heterogeneity of the formulation by decreasing the interfacial tension, Hence, forming homogenous particles with minimum size. The other two variables such as solid lipid: total lipid ratio and

bioactive concentration were positively affecting the PDI. Previously, the increased solid lipid concentration was found to be increasing the particle size of the formulation. This may also increase the PDI of the formulation because of the lower amount of liquid lipid and increased particle heterogeneity due to the presence of some bigger particles. The amount of the bioactive also displayed a positive relationship with the PDI of the NLCs formulation.

3.1.6 Checkpoint analysis

A checkpoint analysis was performed to confirm the prediction. There was an excellent agreement between the measured responses and predicted responses. The experimental values were very close to the predicted values, with low percentage bias, suggesting that the mathematical model was reliable, and hence, the proposed model can be used to navigate the design space as depicted by table 4.

3.2 Evaluation of physicochemical characteristics of optimized NLCs (uncoated), and chitosan-coated NLCs formulation

The average vesicle size of the optimized formulation was found to be 168.24 ± 8.24 nm. The value of the PDI was found to be 0.151 ± 0.003 indicating a homogeneous population. The percentage entrapment efficiency of the optimized formulation was found to be 77.42 ± 3.76 %. The optimized formulation was further coated with 0.2% w/v chitosan solution prepared by dissolving 100 mg chitosan in 50 mL of 1% v/v acetic acid solution. As expected, the particle size of NLCs was found to be increased from their initial size to 317.7 ± 15.9 nm and the PDI was found to be 0.089 ± 0.009 indicating the uniform surface coating of the NLCs. Chitosan coating on the surface of Resveratrol-loaded NLCs is expected to increase the residence time of the formulation into the nasal cavity, due to the mucoadhesive property of chitosan polymer. The chitosan has cationic amino groups in its structure, which interact with the negatively charged mucin into the nasal cavity and form weak ionic bonds, providing the bioadhesive property to the formulation. Chitosan may also enhance the nose-to-brain uptake of the bioactive by interacting with the nasal epithelium. Resveratrol-loaded NLCs (RN), and chitosan-coated resveratrol-loaded NLCs (Ch-RN) were found to be physically stable, free from grittiness, and translucent. The integrity of resveratrol-loaded NLCs and Ch-RN was also confirmed through microscopic studies and TEM analysis. The increased particle size of the formulation may be due to the chitosan coating on the surface of NLCs, and PDI less than 0.1 indicates the homogeneous dispersion of the chitosan-coated formulation. The TEM images of optimized RN and Ch-RN formulations are shown in Figure 5 revealed that the vesicles have a well-identified structure and spherical.

3.3 *In vitro* bioactive release study

The release study was done of the bioactive solution, uncoated NLCs, and chitosan-coated NLCs employing the enhancer cell method with modified USP type 2 dissolution apparatus, and the results are shown in figure 6. The Resveratrol solution has shown release >90% release in 9-10 hr, indicating that the bioactive successfully passing through the dialysis membrane. Both uncoated and coated formulations exhibited the sustained release phenomena by releasing >59% bioactive (uncoated NLCs), and >52% Res.

release (chitosan-coated NLCs) in 24 hr. Both coated and uncoated NLCs have exerted initial burst release followed by the prolonged sustained release of Res. The initial burst release may be because of the untrapped bioactive present on the surface of the formulation. The release rate of Res. from uncoated and coated NLCs was not significantly different. The sustained release characteristic of these formulations may be very important for reducing the dosing frequency and increasing the therapeutic efficacy of Resveratrol. Additionally, the chitosan-coated will increase the residence time of the formulation, and may also increase the nose to brain uptake of NLCs by interacting with the biological membrane of the nasal mucosa. Ultimately, increasing the nose to brain targeting and concentration of the resveratrol into the brain.

3.4 DPPH Assay for antioxidant activity

There are various reports present stating the antioxidant activity of resveratrol. Resveratrol has free radical scavenging activity, hence a DPPH assay was performed on the resveratrol-loaded NLCs for the confirmation of the presence of the same activity in the entrapped form of the bioactive. Sesame oil was used as a liquid lipid in the formulation, which also has reported antioxidant activity. The decreased absorbance of the DPPH solution at 517 nm and the change of color of DPPH solution from purple to yellow was selected as a parameter for the indication and measurement of the antioxidant activity of bioactive solution, and formulations. Similar to the available reports, the concentration of free radicals in the DPPH solution was found to be significantly decreased by the addition of bioactive solution, confirming the antioxidant potential of resveratrol. The free radical scavenging activity of the formulation was found to be higher compared to the bioactive solution, this may be due to the synergistic antioxidant effect of resveratrol, and sesame oil (represented in table 5). Thus, the prepared NLCs did not reduce the antioxidant effect of resveratrol but enhanced the overall antioxidant potential of the formulation.

3.5 *Ex vivo* permeation study

This study was done using the Franz cell diffusion method. The sheep nasal mucosal membrane was selected because of its easy availability, and similarity with the human nasal mucosal membrane. The results from this study demonstrated that the resveratrol from the formulation was permeated slowly compared to the permeation from resveratrol solution, this may be due to the sustained release characteristic of the formulation. The chitosan-coated formulation showed the highest percentage cumulative permeation compared to the uncoated formulation and drug solution as shown in figure 7. This may be because of the interactive ability of chitosan with the negatively charged mucin present on the surface of the nasal mucosa. Furthermore, the chitosan present on the surface of the NLCs may interact with the biological membrane resulting in decreasing the strength of intercellular tight junctions and increased intracellular permeation across the nasal mucosa. Collectively, these mechanisms may be responsible for increased resveratrol permeation across the nasal mucosa by chitosan coated of the carrier system.

4. Conclusion

Despite having huge therapeutic potential, the therapeutic efficacy of resveratrol is limited because of its low bioavailability, low solubility, and short biological half-life. Direct nose-to-brain delivery can be a breakthrough in increasing the bioavailability of resveratrol in the brain, but it also comes with one or more pitfalls such as rapid mucociliary clearance, low administration volume, etc. In this study, the resveratrol-loaded NLCs were prepared and optimized using the Box-Behnken design. The CMAs (solid: total lipid ratio, surfactant concentration, and bioactive amount) were varied between the predetermined range to get the optimum CQAs such as minimum particle size, maximum entrapment efficiency, and minimum PDI. Additionally, this optimized batch was coated with chitosan to facilitate the mucoadhesion and residence time of the formulation inside the nasal cavity. The physiochemical, and morphological evaluation of the coated and uncoated NLCs indicated that the particle size, entrapment efficiency, and PDI were inside the desired range, and the TEM analysis and increased particle size of coated NLCs confirmed the successful chitosan coating on the surface. Furthermore, the in vitro release and ex vivo diffusion studies showed the sustained resveratrol release from the uncoated and coated NLCs, and increased resveratrol diffusion across the nasal mucosa from the coated NLCs. Thus, considering these shreds of evidence, chitosan-coated NLCs can be used to deliver cargos directly into the brain via the nose-to-brain pathway. Although there is still a need for extensive preclinical, biodistribution, and clinical studies to fully prove the therapeutic potential of this carrier system and resveratrol for Glioblastoma multiforme.

Declarations

Ethics approval and consent to participate: Not Applicable

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Tables

Table 1: Variation range of CMAs and required range of CQAs

CMAs	Coded and actual value		
	Low (-1)	Medium (0)	High (+1)
Solid: Total lipid ratio (X1)	0.6	0.7	0.8
Surfactant concentration (X2)	0.5 % w/v	1 % w/v	1.5 % w/v
Amount of bioactive (X3)	10 mg	20 mg	30 mg
CQAs	Target		
Particle size (Y1)	Minimum		
Entrapment efficiency (Y2)	Maximum		
PDI (Y3)	Minimum		

Table 2: Optimization of resveratrol NLCs using Box-Behnken Design

Batch No.	CMAs in coded form			CQAs		
	(X1)	(X2)	(X3)	Particle size (nm) (Y1)	Entrapment efficiency (%) (Y2)	PDI (Y3)
F1	0	0	0	264±9.11	41.4±2.49	0.215±.001
F2	0	0	0	260.3±11.67	42.25±3.66	0.215±.002
F3	-1	0	-1	193.8±5.81	35.7±1.60	0.191±.003
F4	-1	1	0	167.2±5.72	64.24±1.34	0.142±.001
F5	0	0	0	262.7±12.81	40±3.71	0.221±.004
F6	1	1	0	302.8±6.66	33.8±5.46	0.245±.004
F7	1	0	-1	330.6±12.11	25.3±1.73	0.319±.003
F8	0	-1	-1	360.9±4.94	28.5±3.95	0.332±.025
F9	1	-1	0	446.2±10.13	71.87±3.15	0.354±.027
F10	-1	0	1	250.3±10.77	82.4±3.63	0.215±.019
F11	1	0	1	384.2±13.76	77.31±3.46	0.34±.021
F12	-1	-1	0	372.5±6.17	76.52±1.97	0.332±.017
F13	0	1	1	220.6±7.60	69.62±2.60	0.192±.022
F14	0	-1	1	462.5±7.43	73.6±6.78	0.408±.026
F15	0	0	0	255.1±5.69	39.25±5.96	0.207±.021
F16	0	0	0	257.4±18.72	45.35±4.96	0.211±.15
F17	0	1	-1	237.5±11.12	23±2.17	0.197±.016

(All values are reported as mean ± S.D., n=3)

Table 3: Summary of results of regression analysis for responses Y1, Y2 and Y3

Response	Analysis	R ²	Adjusted R ²	Predicted R ²	Std. Dev.	%CV	Model
Y1	Polynomial	0.9944	0.9872	0.9171	9.57	3.23	Quadratic
Y2	Polynomial	0.9829	0.9609	0.7927	0.0019	8.42	Quadratic
Y3	Polynomial	0.9935	0.9851	0.9284	0.1456	3.43	Quadratic

Table 4: Desirability and checkpoint analysis

CMAAs	Solid: total lipid ratio	Surfactant (%)	Amount of bioactive (mg)	Particle size (nm)	Entrapment efficiency (%)	PDI	Desirability
Predicted values	0.6	1.5	22.7	157.23	84.48	0.142	0.998
Observed values				168.24	77.42	0.151	
Percentage prediction error (%)				6.99	8.37	5.6	

Table 5: Percentage DPPH inhibition activity

S. No.	Sample	%DPPH inhibition
1.	Ascorbic acid	96.36948±0.35
2.	Resveratrol solution	68.76182±1.84
3.	Resveratrol loaded NLCs	71.40676±1.22
4.	Blank formulation	20.32507±.48

Figures

Hot-micro-emulsification homogenization method

Figure 1

Method of preparation of resveratrol NLCs

Figure 2

Contour plots and 3D response surface plots showing the effect of factor X1, X2, and X3 on particle size

Figure 3

Contour plots and 3D response surface plots showing the effect of factors X1, X2, and X3 on entrapment efficiency

Figure 4

Contour plots and 3D response surface plots showing the effect of factor X1, X2, and X3 on PDI

Figure 5

(A) TEM image of uncoated resveratrol loaded NLCs (RN), (B) TEM image of chitosan-coated resveratrol loaded NLCs (Ch-RN)

Figure 6
In vitro release profile of resveratrol solution, uncoated NLCs, and coated NLCs

Figure 7

Ex vivo permeation studies of resveratrol from chitosan-coated NLCs, uncoated NLCs, and resveratrol solution