MicroRNA-219 loaded chitosan nanoparticles for treatment of glioblastoma

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ABSTRACT
Recent evidence has implicated microRNA-219 (miR-219) in regulation of gene contributed in glioblastoma (GBM) pathogenesis. This study aimed to prepare miR-219 in chitosan (CS) nanoparticles (NPs), characterize and investigate their efficacy on human GBM cell line (U87 MG). NPs were prepared using ionic gelation method. The influence of process parameters on physicochemical characteristics of NPs was investigated. Apoptotic effect of miR-219 was examined on U87 MG cells. Formulated NPs showed particle size of 109 ± 2.18 nm, with poly dispersity index equal to 0.2 ± 0.05, and zeta potential of +20.5 ± 0.7 mV. Entrapment efficiency of miR-219 in loaded NP has reached 95%. The in vitro release study demonstrated sustained release pattern of miR-219 from CS-NPs. Gel retardation assay has confirmed the integrity of miR-219 after production process. The fabricated NPs reduced the survival of U87 MG cells to 78% after 24 h of post-transfection, and into 67.5% after 48 h. However, fibroblasts were not affected by the NPs, revealing their specificity for GBM cells. Given the tumour suppressing function of miR-219, and advantage of CS-NPs for gene delivery to the central nervous system, the presented NPs have a great potential for treatment of GBM.

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Introduction
Glioblastoma (GBM) is the most common type of brain tumours. It had been classified by World Health Organisation as high-grade glioma, indicating its aggressive metastasis and invasiveness [1,2]. In this type of tumours, cells showing anaplasia, mitotic activity, microvascular proliferation and/or necrosis with poor prognosis [3].

The present standard of care depends on surgery, radiotherapy (RT) and chemotherapy (CT) [4]. This combination therapy fails to extend the survival more than 15 months [5]. The molecular diversity of the disease, along with the presence of cancerous stem cells, lead to the tumour recurrence and poor prognosis [6,7]. Consequently, extensive research efforts have focussed on advanced strategies to target the disease that could significantly improve outcomes of GBM patients [8]. In this regard, microRNAs (miRNAs) have drawn much attention owing to their essential role in biological characteristics of GBM [9,10]. They are 19–24 nucleotides of non-coding RNAs that negatively regulate gene expression post-transcriptionally through binding to complementary sequences on the 3’ untranslated regions (3’UTR) of target mRNAs and typically silence genes [11]. Through deregulation of their target genes, miRNAs paly crucial roles in GBM through several pathways involved in cell proliferation, resistance to apoptosis, autophagy, invasion, metastasis, angiogenesis and drug resistance [3,12].

Based on their biological context, microRNAs have been reported to function as oncogenes or tumour suppressors in GBM [13–15]. In this aspect, it has been found that miRNA-219-5p (miR-219) is one of the downregulated miRNAs in GBM [16,17]. Microarray analysis of brain tissues collected from GBM patients from all stages of the disease revealed a substantial decrease in miR-219 expression compared to normal astrocytes. The study was also carried out on GBM cell lines and revealed reduced expression of miR-219 [16]. Cell proliferation, migration and invasion were all reduced when human GBM cells (U87 MG) were transfected with miR-219 [18–20]. The tumour suppression effect of miR-219 is found to be mediated through epidermal growth factor receptor (EGFR), Sal-like protein 4 (SALL4) and roundabout homolog 1 (ROBO1) proteins [21–23]. In vivo, miRNA-219-5p inhibited tumour growth in nude mouse xenograft models [18]. The findings of these studies suggest the therapeutic potential of miRNA-219-5p in treatment of GBM.

In animal studies convention enhanced delivery (CED), which is a technique based on catheters stereotactically implanted to infuse the therapeutics directly through the interstitial spaces of the central nervous system (CNS) to the tumour site, was utilized to deliver anti-miRNA let-7a into...
the brain of mice xenografted with an aggressive GBM [24]. Administration of anti-miR let-7a by this strategy was efficient to significantly reduce the expression of target gene, a high-mobility group at-hook 2, which is one of the target genes of let-7a [24].

Although direct delivery of miRNA into the brain appears to be effective, non- or less invasive delivery methods would be preferable. However, there are many limitations for administration of miRNAs in vivo because of their susceptibility to RNase degradation, poor stability and poor cellular uptake. Therefore, nanoparticles (NPs) seem a very promising strategy that has been utilized for gene delivery including miRNAs in cancer including GBM [25-30]. Polymeric NPs fabricated from natural polymer such as chitosan (CS), have been utilized to deliver therapeutics including oligonucleotides to the CNS. CS is a naturally derived, biodegradable and biocompatible polymer. It is composed of N-acetyl-D-glucosamine and β-(1,4)-linked D-glucosamine linked by glycosidic bonds. It is polycationic in nature, which allows binding with negatively charged therapeutics, such as miRNA [31,32]. This polycationic nature of CS-NPs will stabilize the genetic material protect it from degradation, and promote its uptake into target cells [31]. In recent study, CS-NPs were prepared and utilized to deliver small interfering RNA (siRNA) intranasally against galectin-1 to GBM GL261 cells, as well as to primary tumour cultures. These NPs resulted in significant reduction in galectin-1 expression in both cell lines [33]. In other in vitro study, CS-tripolyphosphate (TPP) NPs have been fabricated to deliver anti heat shock protein 70 (HSP70) in the GBM U251N cell line, leading to 40% decrease in HSP70 levels with minor toxicity [34]. These studies revealed successful implication of CS NPs as gene delivery in GBM treatment. According to our knowledge, there are no studies yet have developed CS NPs loaded with miR-219. Given the tumour suppressing function of miRNA-219-5-p, and advantage of CS NPs for gene delivery to the CNS, therefore, this study aimed to prepare CS NPs carrier for miR-219 as potential therapeutics for GBM.

Materials and methods

Materials

Low-molecular weight CS (LMW, 50 – 190 kDa, Degree of deacetylation; DD = 75%–85%), sodium TPP and acetic acid were purchased from Sigma-Aldrich (St Louis, USA). miR-219 mimic (UGAUUGUCCAAACGCAAUUCU,) with a molecular weight of 13989.51 g/mol (7281.4 Dalton) and negative control (NC) miRNA were obtained from RiboBio (Guangzhou, China). Quant-iT RiboGreen RNA Assay Kit was purchased from Invitrogen (Carlsbad, CA, USA). Agarose were obtained from MilliporeSigma (Burlington, MA, USA). OmniPurR Ethidium Bromide and OmniPurR DNA Gel Loading Dye 6×, and GeneRuler (50 bp) DNA Ladder were purchased from Thermo Scientific (Waltham, MA, USA).

Methods

Preparation of CNPs

CNP-miR-219 was generated by ionic gelation method as described previously [35]. Solutions of LMW CS was prepared at different concentrations ranging from 0.025% to 0.5% (w/v), dissolved in 1% (v/v) of acetic acid. Then, pH of CS solution was adjusted into five using 10 N NaOH. For addition of miRNA, TPP was pre-incubated with 24 μg of miR-219 or miR-NC for each 2 ml of NPs at different N/P ratio of (50, 100, 150, 200 and 250) before the addition to CS solutions. Then, TPP containing miRNA was added dropwise to the CS solution at optimized parameters under stirring for 1 h at room temperature. After an incubation time of 40 min at 4 °C, NPs were collected by ultracentrifugation using 40,000 × g, at 4 °C for 50 min. The supernatant was conserved for EE% determination. The collected NPs were reconstituted with 400 μl of 12% sucrose, lyophilized and stored for further use.

Entrapment efficiency (EE%)

The miRNA EE% in prepared NPs was determined as previously described using Quant-iT RiboGreen RNA Assay Kit [36]. Briefly, NPs loaded with miRNA were centrifuged at 40,000 × g for 50 min and then, the amount of unentraped miRNA in the supernatant was quantified using RiboGreen® assay. EE% was calculated by following equation:

\[ \text{miRNA EE\%} = 1 - \frac{\text{Amount of free miRNA}}{\text{Amount of entrapped miRNAs}} \times 100 \]

Particle size (PS), polydispersity index (PDI) and zeta potential (ZP) of the formed NPs were measured in triplicate, without further dilutions, at 25 °C using Zetasizer Nano ZS90 (Malvern Instruments Ltd, UK). The samples were measured in phosphate saline buffer (pH 7.4) and each measurement was done in triplicate.

Characterisation of NPs morphology

The morphology of prepared NPs was visualized by transmission electron microscope (TEM) (JEM-1230EX; Tokyo, Japan). One drop of diluted CNPs solution was loaded on a carbon film 300 mesh copper grid, allowing to sit until air-dried. The sample was stained with 1 M uranyl acetate solution for 5 s at 7 °C before viewing on the TEM.

In vitro release study of miR-219 from NPs formulation

CNP-miR-219 were suspended in 1 ml of Tris-EDTA buffer (TE buffer) in RNase free Eppendorf tubes and shaken in water bath (60 rpm) at 37 °C as described previously [37]. At
pre-determined time intervals, the supernatant was collected for analysis and replaced with fresh buffer. The amount of miR-219 released was determined using Quant-iT RiboGreen RNA Assay Kit.

**Gel retardation assay**

**miR-219 integrity.** CNPs were loaded onto a 2% agarose gel which was prepared with Tris/borate/EDTA buffer. The integrity of miR-219 was analysed by gel electrophoresis.

**Enzyme degradation study.** CNPs were incubated with RNase at 37 °C for different time intervals. NPs were loaded onto a 2% agarose gel. Free miR-219 was also incubated with equivalent concentration of RNase and loaded onto the gel.

**Storage stability**

CNPs were resuspended in ultrapure water and stored at 4 °C and 25 °C temperatures. PS, PDI and surface charge were measured every month.

**Transfection/treatment of cell lines**

**Cell line culturing.** Human GBM cell line (U87 MG) was cultured in DMEM (1 g glucose) medium supplemented with 10% FBS and 1% Antibiotic-Antimycotic solution. The cells maintained in an incubator containing 5% CO2 at 37 °C.

**In vitro transfection.** Transfection was performed as previously described [38]. U87 MG cells were seeded (5k per well) onto 96 well tissue culture plates in culture media and left overnight at 37 °C. After reaching 75% confluency, the media was replaced with starvation culture media containing 1% of FBS and kept overnight at 37 °C. In the next day, the cells were treated with CNPs-miR-219 and NPs loaded with negative control miRNA (CNPs-NC). In addition, different concentrations of miR-219 were transfected using GenMute™ TR according to manufacturer’s instructions to compare it with NPs system. The delivery systems and controls were incubated with the cells for 5 h to mediate transfection. After the 5-h treatment time, the media was changed into culture media containing 10% FBS and placed in the incubator for the following 24 h. After that, the cells were pelleted for further analysis.

**Effect of miRNA loaded NPs on cells proliferation.** Cells proliferation was detected using Presto-Blue reagent. CNPs-miR-219 and CNPs-NC were added to GBM cells for 24 and 48 h of post-transfection. After experimental treatment, the reagent was mixed to the media in 1/10 ratio and incubated at 37 °C for 1 h. The fluorescence of transformed Presto-Blue solution in the wells was measured at 570 and 590 nm using microplate reader. Percentage of cell viability was calculated using equation:

$$\text{Cell Viability (\%)} = \frac{F_{\text{of sample}}}{F_{\text{of control}}} \times 100$$

where “F of sample” is the fluorescence of the transformed Presto-Blue in cells incubated with the formula and “F of control” is the fluorescence of transformed Presto-Blue in cells incubated with the CNPs-NC.

**Statistical analysis**

The difference between experimental groups was analysed using Prism 8 (GraphPad), taking $p < .05$ as the lowest acceptable threshold for significance. For two groups, unpaired two-tailed Student’s $t$-test was applied to analyse the difference significance. One-way analysis of variance was used for the comparison between more than two groups followed by Tukey’s test post-hoc test. The results were expressed as averages ± SD.

**Results**

The study was aiming to generate CNPs that able to entrap, protect and deliver miR-219 for GBM treatment. The optimal pharmaceutical goal was to produce NPs with the smallest particle size and overall positive surface charge. Accordingly, the influence of pre-determined parameters that contribute to size and charge of produced NPs were investigated and optimized. These parameters include CS concentration, its pH, its ratio to TPP and weight ratio of NPs to loaded gene.

**Optimisation of prepared CNPs**

**Optimisation of CS concentration**

The concentration of CS played a significant role in the resultant PS and ZP. It is clear from Figure 1(A) that the PS and ZP were increasing with increasing of CS concentration. The selected concentration was 0.05% (0.5 mg/mL), in where PS was of 112.2 ± 2.27 nm, and surface charge equal to 33.9 ± 0.9 mV have been obtained.

**Optimisation of CS solution pH**

The pH of CS had influenced the resultant size and charge of produced NPs as shown in Figure 1(B). CS solution with pH of 5 was chosen fairly to be the optimal value.

**Optimisation of CS: TPP ratio (w/w)**

The increasing of CS:TPP ratio had shifted the formulation into higher particle size and surface charge (Figure 1(C)). Obviously, 3:1 ratio has exhibited the minimum PS with optimum positive charge.

**Quantification of EE%**

**Optimisation of Cs-TPP:miR-219 ratio**

Optimal ratio selection depended on size, charge and EE%. EE% had varied correspondingly with the ratio modification. The statistical analysis among all formulations had resulted in no significant difference ($p = .99$). However, PS and surface charge were also criteria for selection of the optimal condition. Table 1 shows formulations characteristics that referred to ratio manipulation. The effect of manipulation on NPs size and charge is illustrated in Figure 1(D). It exhibited that the nanosized particles of miR-
219 loaded in NPs were enlarged subsequently with greater N/P ratio. Subsequently, 100:1 ratio has been selected based on the minimum particles size and the highest EE%.

**Transmission electron microscope**

The formulations were visualized under TEM. In Figure 2, both miR-219-loaded and empty CNPs showed spherical shapes.

**In vitro release profile of miR-219 from NPs formulation**

Cumulative percentage of released miR-219 was calculated. The entrapped drug had been released from NPs at two stages. In the first day, a rapid release of ~50% of the drug was detected. In the following 6 days, drug release showed a constant and sustained release for more than 90% of the loaded miR-219. Figure 3 illustrates drug release profile of miR-219 from the fabricated NPs.

**Mir-219 integrity**

To demonstrate integrity of entrapped miR-219, NPs were loaded on gel electrophoresis and compared to equal amount of naked miR-219. Gel retardation assay, which is presented in Figure 4(A), shows retardation of miR-219 in the third lane, indicating stability of the gene after production process.

**Enzyme degradation study**

Gel assay showed a stability of miR-219 in the established NPs. Furthermore, non-formulated miR-219 was incubated with equivalent concentration to ensure enzyme degradation of the nucleic acids (Figure 4(B,C)).

**Storage stability**

The formulation was stable for up to one month at 4 °C. Conversely, ambient temperature resulted in size and charge reduction after one month. Table 2 summarizes the consequent effects of different storage conditions on NPs stability for three months.

**Apoptotic effect of CS NPs loaded with miR-219 on GBM cells**

U87 MG GBM cell lines were treated by NPs loaded with miR-219, beside other NPs that were loaded with NC, which is non-functional miRNA. In addition, miR-219 and NC were transfected separately into the cells through Genemute™ TR. Cell proliferation was detected by Presto-blue kit at three concentrations of the miR-219. After 24 h of post-transfection, NPs that were loaded with miR-219 at all doses had reduced cell viability into 78% (p < .05; Figure 5(A)). Another experiment was run for 48 h of post-transfection using a dose of 10 picomol (pmol) from CNPs-miR-219, and CNPs-NC as a control. In Figure 5(B), the results showed a further reduction on the percentage of cell viability into 67.5%.

**Table 1. Influence of CS-TPP:mir-219 ratio on NPs physicochemical properties.**

| CS-TPP: miR-219 | PS (nm) | PDI | ZP (mV) | EE% |
|----------------|--------|-----|---------|-----|
| 250:1          | 252.7 ± 6.1 | 0.468 ± 0.03 | 22.6 ± 0.7 | 93.92 ± 1.2 |
| 200:1          | 174.4 ± 0.4  | 0.28 ± 0.001 | 22.6 ± 0.4  | 92.19 ± 2  |
| 150:1          | 138 ± 0.4   | 0.22 ± 0.041 | 21.4 ± 0.9  | 92.23 ± 2.3 |
| 100:1          | 109.1 ± 2.18 | 0.2 ± 0.003  | 20.5 ± 0.7  | 94.87 ± 1.1 |
| 50:1           | 98.93 ± 1.59 | 0.25 ± 0.001 | 19.8 ± 0.4  | 93.52 ± 2.5 |

PS, PDI, ZP and EE% of the prepared NPs at different N/P ratio. EE% was calculated based on the entrapped and free miR-219 using RiboGreen assay. Expressed as mean ± SD (n = 3).
when compared with the control. MiR-219 in NPs as well as in TR had not suppressed viability of fibroblasts, representing normal cells, which indicates formulation safety and specificity for GBM cells (Figure 5(C)).

Survival data

The Cancer Genome Atlas (TCGA) data was extracted from over 200 patients suffering from GBM disease. The analysis was made through OncoLnc on 4 April 2020 [39]. The OncoLnc database includes survival data for 8647 patients from 21 cancer examination completed by TCGA. Looking at TCGA database, significance was found between miR-219, ROBO1 and SALL4 expressions relevant to GBM. In Figure 6(A), expression of miR-219 in GBM for both live and dead populations is presented. However, high expression for ROBO1 and SALL4 significantly (p-value .0286 and .0498, respectively) corresponded to worse prognosis than patients with low expression of these genes which corresponded to better prognosis (Figure 6(B,C)).

Figure 2. TEM images. Spherical NPs were displayed at 100 and 200 nm scale, empty CNPs and loaded nanoparticles with miR-219. The samples were loaded on mesh carbon films using JEM 1400 instrument.
Discussion

Considering poor survival of GBM and complexity nature of the disease, implication of new therapeutics such as miRNAs is required [40]. Lately, miR-219 was proposed to be a tumour suppressor for GBM, in which several glioma tissues exhibit low levels of miR-219 expression. Ectopic expression of miR-219 has decreased the proliferation, migration and invasion of human GBM cell lines U87 MG, and promotes apoptosis. Such effect mediated through targeting of miR-219 to EGFR, ROBO1 and SALL4 that are involved in the pathogenesis of GBM. Therefore, miR-219 has great potential as a therapeutic agent for GBM [21–23]. Many limitations should be treated for successful clinical application of miRNAs such as susceptibility to RNase degradation, stability and poor cellular uptake. To overcome these barriers, polymeric NPs such as CNPs have been used extensively for gene delivery involving miRNAs [41,42]. CS is a natural cationic polymer, which enhances its complexation with negatively.

Table 2. Storage stability of miR-219 CNPs.

| Time (months) | 0 | 1 | 2 | 3 |
|---------------|---|---|---|---|
| PS (nm)       | 109.1 ± 2.08 | 121.2 ± 1.18 | 185.8 ± 2 | 220 ± 1.4 |
| PDI           | 0.2 ± 0.003 | 0.21 ± 0.001 | 0.38 ± 0.03 | 0.35 ± 0.05 |
| ZP (mV)       | 20.5 ± 0.7 | 21.5 ± 0.5 | 23 ± 0.6 | 25 ± 0.4 |

| Time (months) | 0 | 1 | 2 | 3 |
|---------------|---|---|---|---|
| PS (nm)       | 109.1 ± 2.18 | 96.3 ± 3 | 83.6 ± 1.9 | 69.3 ± 2.1 |
| PDI           | 0.2 ± 0.003 | 0.23 ± 0.05 | 0.21 ± 0.001 | 0.24 ± 0.002 |
| ZP (mV)       | 20.5 ± 0.7 | 20.8 ± 0.5 | 18.6 ± 0.7 | 16.4 ± 0.6 |

PS, PDI and ZP of the formulation along three months storage at 4 °C and 25 °C, NPs were reconstituted with ultrapure water. Measurements represent the mean ± SD, n = 3.
charged miRNAs and improves the stability of genes. Superiority of CS is gained owing to its safety, biodegradability and biocompatibility features [43]. Our fundamental criterion for selection of optimal formulation were the size and the surface charge of the fabricated NPs. Literature reports higher cellular uptake and transfection efficiency of the smaller sized particles [41,44,45]. Furthermore, positive charge of the formulation has higher probability of internalisation as consequence of improved electrostatic binding with cell membrane [42,46,47]. In addition, condensed positive charge could promote escape of NPs from endosomes, which promote intracellular release of drug, thus enhance targets genes silencing [48–51].

Pre-determined parameters were investigated and optimized to qualify a formulation with the smallest PS and a considerable surface charge. CS concentration, its pH and its ratio to TPP were studied being critical process parameters that largely affect size and charge of produced particles. As shown in Figure 1(A), PS was decreasing with decreasing of CS concentration. Lower concentration of CS reduces viscosity of the solution, hence higher solubility and better reaction of CS with TPP, leading to smaller PS [52,53]. Therefore, 0.05% CS concentration has been selected as an optimal solution that gave small PS and adequate positive surface which able to carry miRNAs.

Different pH values of CS solutions have observed a notable effect on PS and ZP. Slightly acidic solution of pH = 5 was fairly chosen as an ideal condition to generate the

**Figure 5.** Cell proliferation assay. (A) Cells viability was assessed on U87 cells using Presto-blue kit 24 hr after transfection, the fluorescence was detected excitation 570 nm and emission 590 nm, cell viability was reduced to 78% (p < .05). CNPs: chitosan nanoparticles, NC: negative control miRNA, TR: transfection reagent. (B) Cell proliferation assay on U87 cells after 48 hours of post-transfection. The cells were treated with a dose of 10 picomol, from CNPs-miR-219, and CNPs-NC as a control. Cell viability was assessed 48 hr post transfection and found to be reduced into 67.5%, (p < .05). CNPs: chitosan nanoparticles, NC: negative control miRNA. (C) Investigation of CNPs-miR-219 toxicity on fibroblasts. Using preto-blue kit. Fibroblasts viability were not changed, p = .3.

**Figure 6.** miR-219, ROBO1, and SALL4 expression in GBM patients. (A) Expression status of miR-219 among dead and alive GBM patients. The red rectangle represents Low expression population of the deceased patients, obtained from TCGA survival database [35]. (B) ROBO1 low expressing patients live longer. Kaplan–Meier curve classifying survival of glioblastoma (GBM) patients based on ROBO1 expression. Low expression cut off is equal or less than 20% of expression while High expression is above 20%. Significantly, obtained from TCGA survival database [35]. (C) SALL4 low expressing patients live longer. Kaplan–Meier curve classifying survival of glioblastoma (GBM) patients based on Sall4 expression. Low expression cut off is equal or less than 20% of expression while High expression is above 20%. Significantly, obtained from TCGA survival database [35].
achieved pre-determined goal, like prior reports [38,54]. Gel N:P ratio. The ratio was optimized to minimize PS and [35,55]. Consequently, 3:1 ratio was selected to obtain the Earlier research has endorsed the effect of CS:TPP ratio on PS demonstrates larger particles along with ratio increasing.

complex, as presented in Figure 1(B), previous research has unstable polyplexes. Even though pH of 7 had generated a trostatic binding between polymer and miR-219 turns into protonation of primary amines is decreased, hence the electrostatic, like hydrogen and hydrophobic bonds [38,54].

Weight ratio of CS to TPP was also optimized. Figure 1(C) demonstrates larger particles along with ratio increasing. Earlier research has endorsed the effect of CS:TPP ratio on PS [35,55]. Consequently, 3:1 ratio was selected to obtain the smallest particles. CNPs were loaded with miR-219 at different N:P ratio. The ratio was optimized to minimize PS and maximize EE%. Obviously, 100:1 ratio has generated NPs that achieved pre-determined goal, like prior reports [38,54]. Gel retardation assay confirms integrity of miR-219 after loading in CNPs (Figure 4(A)). Production process at optimized condition has insured the stability of miR-219. In addition, miR-219 loaded NPs were physically stable for one month at 4 °C. These results are in agreement with Raja et al. study in which CS NPs were formulated, using TPP as cross linker, not others including sodium sulphate and poly-d-glutamic acid sodium salt, and were stable for two weeks [56]. Also, in agreements with previous studies [56,57], these results revealed that TPP’s cross-linking effect exerts its properties as a stabilizer and provokes polymer molecules to create stronger interactions and more stable structures, which are less prone to aggregation. Contrarily, particle size and ZP of our formulation, as well as for the mentioned groups, had been reduced after one month of storage at room temperature. Degradation of NPs is responsible for this reduction. In general, higher temperature possibly will accelerate kinetic motion of NPs [58]. Subsequently, it is recommended to store the prepared NPs at 4 °C for one month along with further assessment of apoptotic and gene silencing effects.

In vitro release profile of the drug was also studied. Release of the drug from designed system has exhibited two distinct stages, started by a rapid release of drug in the first day, followed by a constant release at the rest of days. Obviously, kinetic of drug release is dependent on multiple factors, such as shape and size of NPs, amount of cross linker as well as other process and medium condition [59]. Drug release from CS particles incorporates three different mechanisms: (I) release of drug entrapped in surface of particles, (II) diffusion of matrix and (III) release due to polymer erosion [60]. In most cases, release of drug is subjected to more than one mechanism. Adsorbed drug will be released from particle surface leading to a burst effect, which agrees with the first day release in our formulation. On the other hand, entrapped miRNAs were released by diffusion and erosion mechanisms, assuring sustained release of drug. These finding are also supported by earlier studies [56,58,61,62]. Sustained release of miR-219 could promote a favourable prolonged gene silencing effect [43].

U87 MG cells have been treated with 5, 10 and 15 pmol of the optimized formulation. The highest apoptotic effect is shown with 10 pmol, which reduced the survival to 78% when compared with the control after 24 h of post-transfection (Figure 5(A)). The cell viability was further reduced to 67.5% on average after 48 h (Figure 5(B)), indicating the sustained release characteristic of CNPs. Apoptosis of GBM cell line could be a result of high cellular uptake, successful intracellular release and effective gene silencing. A comprehensive understanding of NPs uptake process by targeted cells is an essential consideration for maximising the effectiveness of delivery. Even though CS-based delivery systems have been broadly studied, the definite pathway of cellular uptake has not been fully understood [63,64]. Many considerations involve in adequate internalisation of NPs, such as physicochemical properties of the system and type of targeted cells [65].

To demonstrate the toxicity and specificity of miR-219, normal cells (fibroblast cells) were treated by the generated NPs. Fibroblasts cells viability has not diminished after treatment with miR-219 through both NPs and TR (Figure 5(C)). Importantly, the used CT and RT show no specificity for GBM tissue. Generalized treatments impair defective and healthy cells at the same time, reducing the tolerance of the therapy. Consequently, the provided treatment approach illustrates more affordable and safe therapy. In addition, Figure 6(A) presented the deceased population of low expression population miR-219 which are the target population that could have been saved by increasing miR-219 expression in order to delay or prevent death. Increasing miR-219 expression will decreases both expression of both ROBO1 and SALL4. This decrease are depicted in the population with prolonged survival (Figure 6(B,C)) when both genes are reduced. This suggests that reversing miR-219 expression from low to high is potentially good for prognosis.

Conclusion
In the current study, CNPs loaded with miR-219 were formulated successfully by the ion gelation method and presented optimal physicochemical characteristics after optimisation of Cs concentrations, Cs to TPP ratio, pH of Cs and N to P ratio. The mean diameter of CNPs containing miR-219 was below 109 nm, with positive ZP and optimal PDI. The encapsulation efficiency was high, integrity of miR-219 was maintained after formulation process and sustained release pattern of miR-219 from CNPS was shown. Moreover, the NPs maintained their stability at 4 °C for at least one month. The produced miR-219 loaded CNPs reduced cell viability of human GBM cells more prominently after 48 h, revealing sustained release properties of the CNPs. Such decrease in cell viability was not observed on normal fibroblast revealing the specificity of miR-219 loaded CNPs to cancerous cells. Therefore, the developed CNPs loaded with miR-219 have promising potential for GBM treatment.
Author contributions

FYA and FSA: Conceptualisation, design of study, acquisition of data, validation and writing original draft. RA: Acquisition of data, analysis of data, visualisation, interpretation of data and writing first draft preparation. BA, MB, QA and HA: Acquisition of data, analysis of data, visualisation and interpretation of data. IA: Resources, visualisation, interpretation of data, writing- reviewing and editing. All authors have read and agreed to the published version of the manuscript.

Disclosure statement

The authors declare no conflict of interest.

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Data availability statement

Data available on request from the authors.

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