Antitumor Activity of Celery Loaded Lipid PLGA-TPGS Nanoparticles in Glioblastoma

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Abstract: Glioblastoma (GBM) is a highly aggressive brain tumor characterized by high recurrence and poor prognosis. Natural compounds are good alternatives for treating glioblastoma and are less toxic than synthetic drugs. Apigenin (AGN) is an effective phytochemical with strong antioxidant and anticancer potential. However, due to its high lipophilicity, the therapeutic effect is limited. Therefore, apigenin lipid polymer hybrid nanoparticles (LPHyNPs) were prepared and characterized according to various parameters in this study. The mean particle size (234.100±23.320 nm), PDI (0.330±0.098), zeta potential (-5.403±0.650 mV), EE (54.99±4.13%), etc. Besides, Morphological analysis by SEM showed that the spherical NP had dark nuclei, indicating that the drug was embedded in the nucleus. In vitro release studies showed that continuous release of AGN from LPHyNPs significantly increased the suspension level of AGN ($P<0.05$). Cell proliferation and apoptosis experiments showed that LPHyNPs could significantly inhibit cell proliferation and promote cell apoptosis. Therefore, the LPHyNPs developed may be an effective therapeutic system for the management of GBM.

Keywords*:* Apigenin; Hybrid Nanoparticles; Glioblastoma; Anti-Cancer Activity

Introduction

Glioma originates from glial cells and is a common malignant tumor in central nervous system. Gliomas can be classified into grade I to IV, with the malignant degree increasing successively, among which glioblastoma (GBM) has the highest malignant degree, with the incidence as high as 46.1%. The average survival time of patients is about 14.6 months, and the 5-year survival rate is less than 5% [1]. The gold standard of treatment for GBM combines surgical excision, radiation, and chemotherapy. However, currently available treatments are so inefficient that they only marginally improve the average survival rate of patients [2]. Therefore, the search for novel approaches to GBM, therapy with reduced adverse reactions is still necessary.

Apigenin (AGN) is a natural flavonoid found in a variety of vegetables and fruits, as well as some medicinal plants [3]. A number of studies have shown that AGN has a variety of therapeutic potential, such as anti-cancer, anti-inflammatory, antioxidant, anti-diabetes, etc[4, 5]. However, due to its high lipophilicity, its therapeutic effect is limited [6]. Over the past few decades, nanoparticles, such as lipid- and polymer-based systems, have made significant advances in targeted drug delivery. Nanotechnology offers an effective solution for treating different diseases because it has the potential to improve drug delivery throughout the body and increase their bioavailability [7, 8]. In addition, nanoparticles can be designed to selectively target specific cell receptors, thereby enhancing their absorption or facilitating their penetration through BBB [9]。

In this study, Vit-E-TPGS lipids HyNPs (LPHyNPs) containing AgNs were synthesized and characterized. It was subsequently validated for its role in GBM cells, aiming to provide a safe and effective drug for the treatment of GBM.

1. Materials and methods

1.1 Materials

AGN, Poly (lactic-co-glycolic acid) copolymer PLGA; 50:50, molecular weight 55000, Jinan Daigang Bioengineering Co., Ltd.). Polyvinyl alcohol (88% hydrolysis degree, Shanghai Weicheng Chemical Co., Ltd.). Both methanol and acetone were purchased from Sigma Aldrich (St. Louis, USA). D-alpha-tocopherol polyethylene Glycol 1000 succinate (Vit E TPGS) was obtained from Sigma Aldrich, St. Louis, Missouri, USA. Moreover, Lipoid SPC (hydrophosphatidylcholine from soy) was bought from LIPOID (GmbH, Ludwigshafen Germany). Dichloromethane (DCM) and dimethyl sulfoxide (DMSO, 99.9%) were got from Fischer Scientific (Loughborough,UK). GBM cell line U251 was obtained from the Cell Bank/Stem Cell Bank of the Chinese Academy of Sciences (Shanghai, China), while Annexin V/PI Apoptosis Assay kit was purchased from Invitrogen Corporation (Carlsbad, CA, USA).

1.2 Preparation of LPHyNPs nanoparticles

LPHyNPs were prepared by nanoprecipitation method[10] with slight modification. In briefly, 50 mg PLGA (50:50), 100 mg Lipoid S PC-3, and 5 mg AGN (previously dissolved in 100 µL DMSO) were dissolved in 5 mL DCM in first stage. In the second stage, the AGN/PLGA weight ratio was maintained at 1:10 w/w and the lipid: PLGA weight ratio was 2:1w/w. The Vit E-TPGS 1000 was dispersed at 0.5% w/v in 10 mL Milli-Q water heated to 70°C. The first stage solution was then added drop by drop (at a rate of 1.5 mL/min) to the preheated second stage solution at magnetic agitation (500 rpm). The mixed solution was then homogenized (T25 digital Ultra-Turrax, IKA, UK) for 2 minutes (21,000 rpm) and magnetically stirred at 25±1 °C at 500 rpm for 4 hours to completely evaporate the DCM. The final preparation is washed with Milli-Q water by centrifugation at 30,000 rpm at a high speed for 30 minutes (three cycles). The prepared LPHyNP was recovered and purified using dialysis techniques (Spectra/PorVR dialysis membranes). The final LPHyNPs formulation (100 µL) was diluted 50 times with Milli-Q water and characterized by dynamic light scattering (DLS) measurements, such as particle size, PDI, and zeta potential. In addition, mannitol (1%, w/v) was used as a freeze protector and frozen at -80°C and freeze-dried for further characterization.

1.3 Characterization of LPHyNPs

1.3.1 Mean Particle Size (Z-Ave), zeta Potential and Polydispersity Index (PDI)

The mean particle size and zeta potential of nanoparticles were measured by dynamic light scattering and electrophoretic light scattering techniques [11]. At the same time, the polydispersity index of the nanoparticles was measured, which showed that the particle size distribution of the nanoparticles was uniform. The freshly prepared NP formula was diluted appropriately before measurement. Three measurements were taken using Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK).

1.3.2 Determination of encapsulation efficiency and drug loading

Drug embedding (EE) and loading efficiency (DL) of prepared LPHyNP were analyzed in supernatant of collected samples (indirect method)[12]. Approximately 5 mL of LPHyNPs was diluted in 5 mL of methanol to dissolve the drug and precipitate PLGA and other excipients. The suspension (centrifuge at rpm for 20 minutes at 4 ° C) was centrifuged and the supernatant was collected. To analyze drug concentrations in the collected supernatant, a 30 µL sample was injected into an HPLC-UV system. A chromatographic technique containing a C18 column (5 µm, 250 mm × 4.6 mm) was developed for this purpose. The mobile phase consisted of acetonitrile and 0.1% formic acid at 55:45 (v/v), where the pH was maintained at 7.4, and was isodemi- pumped at a flow rate of 1 mL/min and UV detection was performed at 270 nm. According to the following equation:

Encapsulation rate calculation formula：EE%= Measured drug load/Theoretical drug load×100%.

Formula for calculating drug load：DL%= The total amount of drug measured in the nanoparticles / Total mass of nanoparticles×100%.

1.3.3 Morphological determination

The shape and surface morphology of apigenin loaded nanoparticles were determined by scanning electron microscopy (SEM). SEM micrographs of nanoparticles were obtained by Au coating at 7.00kV.

1.4 In vitro release studies

In vitro drug release was performed using dialysis bags with an interception molecular weight of 12,000Da. In short, the LPHyNPs suspensions were placed separately in dialysis bags, knotted and dipped into the release medium. The release medium (50 mL) was phosphate buffered brine (PBS) with pH 7.8 and sodium dodecyl sulfate (1%) as the solvent enhancer. Throughout the process, the system was maintained at 37 °C in a shaker bath. Samples were collected at 1, 2, 3, 4, 6, 8, 24, 48, and 72 h, and AGN content was analyzed at given time intervals using HPLC techniques.

1.5 Cell activity assay

The growth inhibitory activity of LPHyNPs on U251 glioma cells was determined by MTT assay. U251 glioma cells were inoculated in 96-well plates with a density of 1×10^4 cells/well, and incubated overnight at 37℃ and 5% CO_2. The cells were treated with blank NP, AGN, and LPHyNPs at concentrations of 6.25, 25, and 100 μg/mL and left for 24 h. Then, 50 mg/ml MTT reagent was added to the sample and incubated for another 3 h until the purple product was formed. Formaldehyde crystals formed in 0.1ml DMSOlyzed cells were added, and absorbance was measured at 570 nm using an enzyme label.

1.6 Cell apoptosis assay

Apoptosis was determined using the annexin V (FITC)/PI assay kit (K101-100, Biovision Inc., Milpitas, CA, USA). In simple terms, 1×10^5 cells /mL were inoculated into 96-well plates, incubated for 24 h, then centrifuged, washed with phosphate buffers, and resuspended with 500μL buffers. Subsequently, 100μL of the resuspension cells were incubated again with 5μL PI and Annexin-V in a darkroom at room temperature for 15 min. The data were analyzed using a BD FACSCalibur reader and flow cytometry and flow system software.

1.7 Statistical analysis

The data were processed by SPSS 26.0 and Graphpad prsim 9 software. Measurement data were expressed as mean ± standard deviation and counting data as %. T test or one-way analysis of variance were used to compare the differences between groups. $P<0.05$ indicated significant difference and had statistical significance.

2. Results

2.1 Differences in mean particle size, zeta potential and polydispersity index of LPHyNPs

As shown in Table 1, the average particle sizes of prepared blank LPHyNPs and loaded LPHyNPs were 201.100±10.090 nm and 234.100±23.320 nm, respectively. Meanwhile, the PDI of blank LPHyNPs and loaded LPHyNPs were 0.113±0.061 and 0.330±0.098, respectively. These results were related only to the addition of drugs. After the addition of the drug, the particle size of NP increases due to the inclusion of the drug, which enlarges the particle size. On the contrary, PDI of loaded LPHyNPs was decreased compared with blank LPHyNPs, indicating a uniform amount of NP. In addition, the average zeta potential of blank LPHyNPs and loaded LPHyNPs were -4.277±0.968 and -5.403±0.650, respectively (Figure. 1).

Table 1. Average particle size, zeta potential and polydispersity index of LPHyNPs

Groups	mean particle size (nm)	zeta potential (mV)	PDI
Blank LPHyNPs	201.100±10.090	-4.277±0.968	0.113±0.061
LPHyNPs	234.100±23.320	-5.403±0.650	0.330±0.098

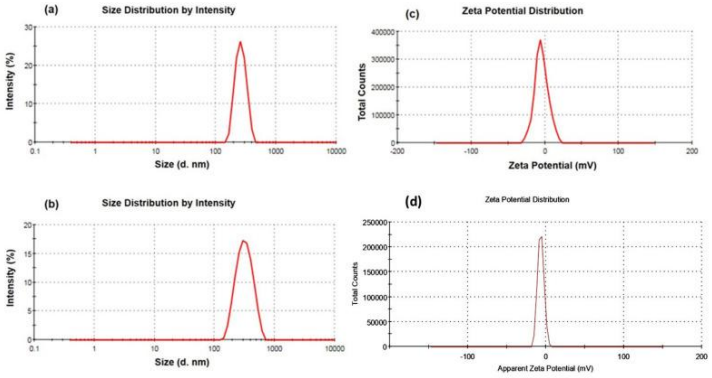

Figure 1. (a; b) Particle size of blank LPHyNP and loaded LPHyNP, and (c, d).zeta potential of blank LPHyNP and loaded LPHyNP

2.2 Drug encapsulation and loading efficiency for preparing LPHyNPs

EE and DL of LPHyNPs were calculated to determine drug concentrations in NP. The EE of LPHyNPs was 54.99±4.13%. In this case, the drug is usually encased in NP, thereby continuously releasing the drug from LPHyNPs. At the same time, DL of LPHyNPs was recorded as 11.13±0.83%, DL due to the affinity of AgNs to the polymer used (i.e., PLGA).

2.3 Morphological analysis of LPHyNPs

The surface morphology of apigenin nanoparticles was observed by scanning electron microscopy at 7.00 kV. According to the SEM micrographs obtained, the nanoparticles were spherical and the surface morphology was smooth (Figure 2).

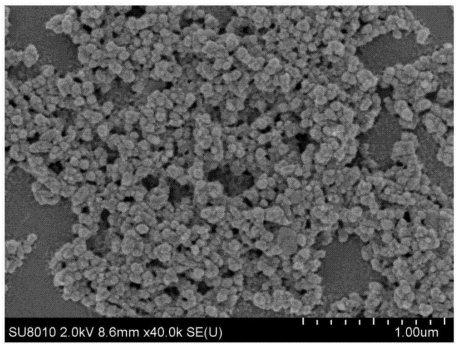

Figure 2. SEM image of LPHyNP

2.4 Study on release patterns in vitro

In vitro drug release study was carried out by dialysis bag method, and the release mode of LPHyNPs and AGN suspensions was analyzed. The results were shown in Figure 3, and the release of AGN from LPHyNPs was sufficient to last 72 hours. In contrast, AGNs are fully released within 24 hours, with a rapid release pattern of AGN suspension. In the case of AGN release from LPHyNPs, the initial burst release was observed for up to 8 hours and then continued for up to 72 hours. Therefore, the release of AGN by LPHyNPs was significantly better than that by AGN suspension ($p<0.05$). LPHyNPs' PLGA package controls release and provides continuous release mode for over 64 hours.

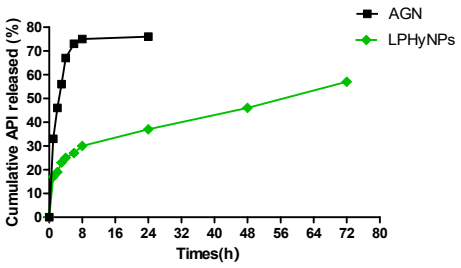

Figure 3. In vitro release of AGN from LPHyNP and AGN by dialysis bag method in a PBS suspension containing SLS (1%) as a solvent enhancer.

2.5 Cell viability analysis (MTT assay)

Biosafety and cytotoxicity of blank LPHyNPs, AGN suspensions, and LPHyNPs were evaluated on U251. The consequences showed that when U251 cells were treated with various drug preparations, the IC50 of blank LPHyNPs was 124.50± 8.34μg /mL, and the IC50 of AGN suspension was 51.37±3.06 μg/mL. LPHyNPs showed an IC50 of 11.05±1.38 μg/mL (Figure 4).

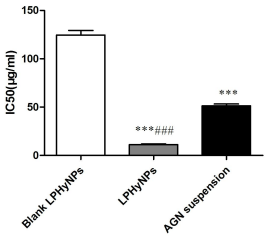

Figure 4. MTT determination results of blank LPHyNP, AGN suspension and LPHyNP. Values are expressed as mean ±SD, ***p< 0.001.

2.6 Cell apoptosis assay

As shown in Figure 5, the apoptotic activity of cells treated with LPHyNP was almost twice that of blank LPHyNP and AGN. On the other hand, blank LPHyNP and AGN showed similar apoptotic activity (Q4:11.08%, blank LPHyNP; 13.51%, AGN suspension) and 24.15% (LPHyNPs). Based on apoptotic activity, it was found that blank NPs showed higher apoptotic activity than the control.

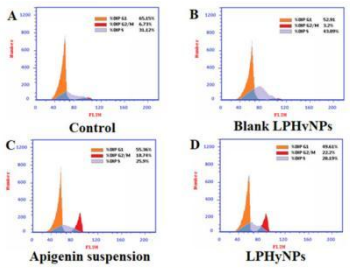

Figure 5. Apoptosis was detected by flow cytometry. (A) Control, (B) blank LPHyNP, (C) apigenin suspension, (D) LPHyNP. Values are expressed as mean ±SD, *p<0.05, **p<0.001. ns indicated a non-significant difference between the results.

3. Discussion

Glioma is a common intracranial malignant tumor of human central nervous system. It is aggressive and has a poor prognosis[13]. BBB and BBTB are the biggest obstacles to the delivery of many drugs to gliomas, thus limiting their

effectiveness. Therefore, the treatment of glioblastoma urgently requires the development of therapeutic drugs and delivery systems that can transcend physiological and pathological barriers.

AGN is a flavonoid widely found in nature, which is mainly extracted from celery, parsley, thyme, chamomile and onion [14]. Studies have shown that it has important antioxidant [15]、anti-inflammatory[16]、anti-tumor[17-20] and anti-fibrosis effects. Although AGN is an effective anticancer molecule, it has significant pharmacokinetic limitations. AGNs have been classified as Class II drugs under the Biopharmaceutical Classification System, which has high permeability and low solubility [21]. Nanoparticles are one of the best ways to improve the solubility and efficacy of low solubility bioactive drugs [22]. Compared with traditional Chinese medicine, nano Chinese medicine has the characteristics of slow release, targeting and high bioavailability. Nano Chinese medicine and nano carrier are prepared into sustained-release agents by various methods, so that the drug carrier complex enters the body in a certain way, and the drug is released from the nano capsule through leach, penetration and diffusion of the wall capsule, avoiding the "sudden release effect". In addition, nanocarriers can resist the degradation of drugs by drug degrading enzymes, play a protective role on drugs, improve the half-life of drugs, and prolong the action time of drugs.

Apigenin lipid polymer hybrid nanoparticles (LPHyNPs) were synthesized and characterized in this study. The mean particle size and PDI of LPHyNPs were 234.100±23.320 nm and 0.330±0.098, respectively, which were significantly higher than that of blank control. The average zeta potential of LPHyNPs was lower than that of the blank control group, indicating that the synthesis of nanoparticles was related to the addition of relevant AGNs. After the addition of AGN, the particle size of NP increases due to the inclusion of drugs, which makes its particle size expand. Therefore, the LPHyNPs developed is considered to be a stable preparation. Moreover, morphological analysis showed that the nanoparticles were spherical with smooth surface morphology.

EE is one of the main advantages of developing PLHNPs. A high EE percentage of PLHNPs is required to achieve the desired therapeutic effect. In this study, EE and DL of LPHyNPs were calculated to determine drug concentrations in NP. The EE of LPHyNPs was 54.99±4.13%. In this case, the drug is usually encased in NP, thereby continuously releasing the drug from LPHyNPs. At the same time, DL of LPHyNPs was recorded as 11.13±0.83%, DL due to the affinity of AgNs to the polymer used (i.e., PLGA).

In vitro release studies showed that the PLGA package of LPHyNPs controlled release and provided a sustained release mode of over 64 hours, which might be due to the existence of a certain amount of free apigenin in the nanostructured lipid carrier, or it might be related to the easy release of drugs adsorbed on the superficial surface or surface of the nanostructured lipid carrier [2].

Antitumor experiments showed that LPHyNPs significantly inhibited the proliferation of glioma blasts, promoted their apoptosis, and showed high anticancer activity, which may be due to the excellent drug penetration of the manufactured nanopreparations into tumor cells. LPHyNP has the potential and effectiveness to improve apoptosis of glioma cells by stimulating apoptotic activity.

In summary, AGN-LPHNPs was prepared by single step nano precipitation method in this study, and showed good stability. Encapsulation of AGNs in LPHNPs improves solubility and represented slow release for up to 72 hours. In vitro experiments confirmed that AGN-LPHNPs has a significant effect on the proliferation and apoptosis of glioma blast cells, and may affect the development of glioma blast cells by inhibiting proliferation and promoting apoptosis. However, this study also has some limitations, and the mechanism of action and in vivo effects of AGN-LPHNPs still need to be further studied.

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