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Fabrication of gemcitabine and losmapimod-prodrug loaded nanoparticles for the treatment and care of lung cancer

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Abstract

Lung carcinoma (LC) is rare cancer in most parts of the world but is common cancer in southern Asia. Local recurrent disease and distant metastasis of LC are still mysterious challenges. This investigation was to effectively fabricate and evaluate the therapeutic efficiency of Gemcitabine (GEM) and Losmapimod (LOS) encapsulated into polyethylene glycol (PEG)-polylac-tico-co-glycolic acid (PLGA)-NPs (GEM/LOS@NPs), which exhibited inhibition of in vitro lung cells proliferation. GEM/LOS@NPs with a diameter of ~100 nm have been constructed employing an enhanced double-emulsion (W/O/W) method. The GEM/LOS@NPs’ particle size was studied through transmission electron microscopic (TEM) techniques and dynamic light scattering (DLS) measurement. After successfully fabricating GEM/LOS@NPs, the anticancer ability of A549 and H460 lung cancer cells was investigated. Biochemical assays such as AO/EB (acridine bromide/orange ethidium) and nuclear staining investigated the cancer cell’s morphology and death. The cell death of the cancer cells was synergically examined by ROS analysis. Overall, these results suggest that highly toxic drugs can be rationally transformed into self-deliverable and pharmacologically efficient monotherapy.

1. Introduction

Lung carcinoma (LC) is the most widespread form of cancer in the world (12.3% of all cancers), with an estimated 1.5 million new cases in 2020 [1]. Tobacco smoking is the most significant cause of lung cancers, with 80%–90% occurring in cigarette smokers [2–4]. There are substantial gender changes, racial geography, and incidence, and several reports imply that women may be at heightened risk of lung cancer from exposure to tobacco smoke carcinogens [5]. Ethnic and regional differences influence the prevalence of LC [6]. Most of the cases occur in Southeast Asia (67%), southern China (45%), and North America (5%). Two to three times more men are diagnosed with LC than women; the incidence peaks between 50 and 60 years of age, and death is observed above the age of 85. Undifferentiated carcinoma, squamous cell carcinoma (affecting adults), and non-keratinizing carcinoma are the three subtypes of LC that have been recognized by the World Health Organization (WHO) (primarily found in children) [2]. To date, several different nanoparticles have been developed to treat LC. Polymeric nanoparticles have been shown in a recent study to slow down the respiration rate of LC cells. When lipid nanoparticles and an LC-specific therapeutic protein were combined, tumor growth was dramatically reduced, the therapeutic effectiveness was enhanced, and the survival rate was raised [7].

As a prodrug, the single method by which gemcitabine works is through intracellular phosphorylation, resulting in the drug’s active triphosphate form [8–10]. Deamination of gemcitabine (GEM) triphosphate (dFdCTP) to 2′,2′-difluorodeoxyuridine (dFdU), a gemcitabine derivative with poor anti-tumor efficacy, is responsible for around 90% of its fast elimination. The gemcitabine’s limited clinical effectiveness and quick metabolism are assumed to be responsible for its short half-life (32–84 min for brief infusions in humans). Since the formulation of gemcitabine had been improved by conjugating long fatty acid chains onto it, new approaches were sought to enhance its lipophilicity [11]. A fatty acid ester derivative of gemcitabine (CP-4126, gemcitabine-5′-elaidic acid ester) showed higher antitumor efficacy than its parent chemical when administered
from vacuum evaporation of dichloromethane.

Polyethylene Glycol-Mw

2. Materials

2.1. Materials

The nanoparticles’ particle size and zeta potential were characterized using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The surface morphology of NPs was examined under transmission electron microscopy (TEM). For the TEM analysis, 10 μl of 1 mg mL$^{-1}$ N$^{-1}$P$^{-1}$ s$^{-1}$ resuspended in Milli-Q was kept on the copper grid for 1.5 min, and then the excess sample was wiped out. After that, 10 μl of 1% (w/v) uranyl acetate was added to the grid for 1 min, and then excess sample was removed. After air drying for 5 min, the TEM grids were placed on a TEM microscopy system for imaging.

2.2. Fabrication of the tri-block GEM/LOS@NPs

Double water–emulsion (W/O/W) GEM loading with minor changes in the PLGA-PEG NPs [31, 32]. 200 mg of PLGA-PEG were dissolved in 5 ml of dichloromethane (DCM). An ultrasonic emulsification procedure utilizing a Sonoplus, HD 2070 (Bändern Elektronik, Berlin) and 5 min at 55% power emulsified the GEM in 25 ml polyvinyl alcohol (PVA) in the organic phase. Centrifugation of the GEM-NPs at 15,000 rpm for 40 min resulted from vacuum evaporation of dichloromethane (DCM) at 40 °C.

The double emulsion (W/O/W) technique was used to develop LOS-NPs. After combining 250 mg of PLGA-PEG in dichloromethane (DCM), 25 mg of LOS was added in the same tube, and the mixture was combined for 15 min at 28 °C with a magnetic stirrer. The PVA emulsion and the 2.5 ml of PVA solution were blended by an ultrasonic examination at 60% for 3 min to make a homogenous mixture. Centrifuged at 15,000 RPM for 40 min, the LOS-NPs were separated from the DCM and washed three times with a water solution.

The double emulsion (W/O/W) method was also used to fabricate GEM/LOS@NPs. After the first emulsion was obtained, it was homogenized for 5 min at 28 °C with 2 ml of PVA. Emulsified for 5 min, the mixture was then re-emulsified with 0.2% LOS-dissolved in DCM. All the work was done in the same way as when making GEM-NPs.

2.3. Characterization of the nanoparticles

The nanoparticles’ particle size and zeta potential were characterized using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The surface morphology of NPs was examined under transmission electron microscopy (TEM). For the TEM analysis, 10 μl of 1 mg mL$^{-1}$ N$^{-1}$P$^{-1}$ s$^{-1}$ resuspended in Milli-Q was kept on the copper grid for 1.5 min, and then the excess sample was wiped out. After that, 10 μl of 1% (w/v) uranyl acetate was added to the grid for 1 min, and then excess sample was removed. After air drying for 5 min, the TEM grids were placed on a TEM microscopy system for imaging.
acetate solution was used to stain the sample coated on a grid for 30 s. Finally, the stained sample was observed at 80 kV under TEM (FEI Company, Hillsboro, OR). All the data were repeated at least three times.

2.4. Encapsulation efficiency (EE) and drug loading (DL)
To quantify the amount of non-entrapped drugs in the tube’s supernatant following the fabrication of dual drug-loaded nanoparticles, we determined the efficacy of drug encapsulation (237 and 288 nm, respectively). Drug loading (DL) and Encapsulation Efficiency were calculated using the following formulae in the following [33]:

\[
\text{DC(\%)} = \frac{\text{weight of GEM/LOS NPs/initial weight of GEM/LOS NPs}}{100}
\]

\[
\text{EE(\%)} = \frac{\text{weight of GEM/LOS NPs/formulation amount of GEM/LOS NPs}}{100}
\]

2.5. In vitro drug release study
PLGA-PEG NPs (25 mg) were dispersed in PBS (5 ml), pH 7.4, then put in a dialysis tube, agitated at 200 rpm, and incubated at 37 °C for 24 h to determine the in vitro release of GEM and LOS release from the GEM/LOS@NPs. The environmental buffer solution was replenished with a new PBS solution at predetermined intervals. UV spectrophotometry (Shimadzu, Japan) was used to measure the concentration of the free drugs at their highest wavelength absorbance and produce respective standard curves [34–36].

2.6. MTT assay
Human lung cancer (A549, and H460) and non-cancerous NIH-3T3 and L929 cells were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (CAS, Shanghai, China). All the cells were grown as a monolayer in a humid incubator in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 95% air, 5% CO2. The MTT test was used to determine both free drugs and drugs loaded with PLGA-PEG for 72 h after exposure. Cell attachment was allowed by incubating at 37 °C for 24 h with humidified 5% CO2 in 96-well plates containing 2 × 104 cells per well. The cell viability of the PLGA-PEG-NPs was examined using non-cancerous NIH-3T3 and L929 cells. Next, the A549 and H460 cells were treated to free GEM, free LOS, GEM@NPs, LOS@NPs, and GEM/LOS@NPs were exposed at varying concentrations (50, 25, 12.5, 6.125, 3.12, and 1.56 μM, respectively). A phosphate-buffer solution containing 0.5 mg/ml of MTT was added to all wells after 48 h of incubation. The plates were covered with aluminum foil and incubated for 4 h at 37 °C. This was followed by removing all the well’s contents with DMSO and 25 μl Sorensen’s Glycine buffer and extracting the produced formazan crystals for 20 min. Finally, we tested the cell lines’ survival following the various treatments utilizing an ELISA-microplate reader set to the formazan maximum absorbance wavelengths (475 nm) [37–41]. All the trials were repeated three times.

2.7. AO/EB (acridine orange/ethidium bromide) staining assay
Fluorescent microscope images of samples stained against A549 and H460 cells with dual acridine orange/ethidium bromide (AO/EB) fluorescence staining were obtained. 2 × 10^3 cells were grown onto 6-well culture plates and incubated for 24 h. After that, cells were left untreated or treated with the IC_{50} concentration of the GEM@NPs, LOS@NPs, and GEM/LOS@NPs for about 24 h. A dual fluorescent AO/EB staining solution (Sigma-Aldrich, USA) (10 g ml⁻¹ working concentration) incubates each well at 37 °C for 10 min following treatment. After that, A PBS wash was performed to eliminate any excess color [42–45]. The dual AO/EB staining procedure was repeated at least thrice.

2.8. Hoechst 33342 nuclear staining
To monitor cytoskeletal alterations and nuclear chromatin compaction, fluorescent dyes Hoechst 33342 were added to A549 and H460 cell lines. 2 × 10^5 cells per well of A549 and H460 cells were seeded in 48-well plates and treated with GEM@NPs, LOS@NPs, and GEM/LOS@NPs for 24 h, respectively. To eliminate the dead floating cells, different incubation times were followed by adding 2 μl Hoechst dye for 15 min at 37 °C to eradicate the dead floating cells [46–48]. Fluorescent dye nuclear morphological assessment was conducted at least three times.

2.9. Intracellular ROS generation
To identify ROS generation with fluorescent probes, we used flow cytometry to verify the redox state of A549 and H460 cells. For assessing oxidative stress in A549 and H460 cells, we used the DCFH-DA method, which has several advantages over other approaches, including being simple to execute and analyze and being particularly
sensitive to changes in a cell’s redox state over time. GEM@NPs, LOS@NPs, and GEM/LOS@NPs were treated with A549 and H460 cells in a 6-well plate at a concentration of \( IC_{50} \), for 12 h. 5(6)-Carboxy-2',7'-dichlorofluorescein diacetate, a peroxide-sensitive fluorescent probe, was used to quantify intracellular ROS (H$_2$DCFDDA; Sigma-Aldrich). The cells were then incubated for 10 min at 37 °C with a dye solution containing 25 μM DCFH-DA dispersed in 1 ml fresh DMEM. Extracted cells were resuspended in sterile PBS after incubation. After incubation, samples were examined under a fluorescence microscope [49–51]. Fluorescent dye ROS assessment was performed at least thrice.

3. Results and discussion

3.1. Characterizations of triblock GEM/LOS@NPs

Over the past few decades, various natural and synthetic polymers have been progressively used in medicinal applications, mainly as drug delivery nanoparticulate systems. Due to its superior biocompatibility and biodegradability, PLGA has been widely studied for developing devices to deliver potential molecules with encouraging results, combined with other biopolymeric materials or alone, because of its broad range of erosion periods flexible mechanical structures [32]. It has been shown that PLGA NPs can be surface functionalized with other biopolymeric materials, such as PEG and PVA, to considerably enhance their blood circulation time, targeting approach, and biocompatibility [53]. Furthermore, PEGylation boosts PLGA NPs, resulting in constructing a stealth particle with a longer half-life in the blood and an improved ability to pass past the digestive system [54]. As a result, in this study, we loaded GEM and LOS using a tri-block copolymer of PLGA-PEG-PLGA.

3.2. Characterizations of combinational drug-loaded NMs

To make polymeric nanoparticles with therapeutic compounds embedded inside, researchers used the water-in-oil-in-water (W/O/W) emulsion process. A key hurdle in creating a nanocarrier-mediated co-delivery system for both LOS and GEM simultaneous application was their varying degrees of water solubility. The W/O emulsion is first prepared for loading biomolecules. Then a W/O/W emulsion in water is produced using a surfactant to generate NPs in the second step of the double emulsion approach. This method has encapsulated both hydrophilic and hydrophobic drug compounds into NPs. This investigation used the enhanced W/O/W system to obtain the co-loaded LOS/GEM PLGA-PEG NPs using the enhanced W/O/W approach (figure 1).

Immune systems should not recognize or destroy the amphiphilic PLGA-PEG copolymers, so their size should be kept to a minimum to maximize the drugs’ lifespan. The diameter and size distribution of NPs were measured using a zeta potential analyzer and the DLS measurement. (figures 2(A)–(C)). The figures 2(D)–(F) shows that the particle diameter of 68.21 ± 7.47, 69.55 ± 5.26, 92.54 ± 6.48 nm for GEM@NPs, LOS@NPs, and LOS@GEM-NPs, respectively, a polydispersive index (PDI) of 0.124 ± 0.004, 0.121 ± 0.003, 0.105 ± 0.003 for GEM@NPs, LOS@NPs, and LOS@GEM-NPs, respectively, and zeta potential of −3.6 ± 0.05, −5.6 ± 0.05, −8.2 ± 0.07 +/− mV for GEM@NPs, LOS@NPs, and LOS@GEM-NPs, all of which were well-organized and uniformly distributed. Further, there was an average size difference between drug-free and drug-loaded nanoparticles, which suggests that the NPs are loaded with drugs in the core, which results in a larger diameter. Nanoparticle size and shape were studied further using TEM (figures 2(A)–(C)). According to
the representative electron micrograph, the LOS@GEM-NPs were evenly dispersed, with 92.54 ± 6.48 nm. For TEM examination, materials must be thoroughly dehydrated, but for DLS, they must be completely hydrated. This minor difference in NP size has been related to this surface modification. Cancer cells can uptake the nanoparticles with diameters smaller than 100 nm. Furthermore, NPs of sufficient size are required for efficient endocytosis into cancer cells and avid macrophage arresting. These physical and chemical features suggest that the loaded LOS/GEM (PLGA-PEG NPs) are potential candidates for internalization into cancer cells. In addition, the stability of the nanoparticles was measured by the particle size, polydispersity index (PDI), and zeta potential of the NPs by the DLS analysis in the aqueous solution (figures 3(A)–(C)) and DMEM cell culture media (figures 3(D)–(F)), which was shows slight changes.

The short- and long-term biostability of NPs and the interaction between NPs and cell membranes are strongly influenced by their surface charge (measured by the zeta potential). The synthesized NPs had an average surface charge of $-3.6 \pm 0.05$, $-5.6 \pm 0.05$, $-8.2 \pm 0.07$ /- mV. The free carboxyl groups in PLGA deprotonate, resulting in the polymer chain’s negative zeta potential values. The zeta potential of smaller mean diameter NPs is higher than that of more significant NPs, making them more stable in colloidal dispersions due to their faster migration velocity under a known applied electric field.

3.3. Assessment of drug release profile

The dialysis membrane approach was used to investigate the drug release profile of LOS and GEM from the PLGA-PEG NPs at pH 7.4. All the drug-loaded formulations revealed similar release profiles. According to figure 4, a quick initial release of both drugs was detected in the first 8 h, resulting in a drug removal over day 12 with close to 70% and 87% liberation of both LOS and GEM within three days. Differences in hydrophobicity values may explain LOS and GEM discharge discrepancies from LOS/GEM-loaded NPs. A similar release pattern has been reported for other PLGA-PEG nanoparticles previously.

3.4. MTT analysis

MTT test was used to examine the combination cytotoxic efficacy of LOS and GEM drug encapsulated from PLGA-PEG NPs. To enhance drug effectiveness while ensuring carrier safety for healthy cells, PLGA-PEG NPs for chemotherapy hold excellent promise. After conducting MTT analysis in NIH-3T3 and L929 cells, PLGA-PEG NPs were determined. Even at a 1.0 μM dosage, PLGA-PEG NPs had no apparent cytotoxicity on NIH-3T3 and L929 non-cancerous cells (figure 5(A)), which reveals that the NPs highly efficient in using potential nanocarriers for various cancer treatments. As illustrated in figures 5(B)–(C), free drugs and combined drug-loaded nanoparticles had a dose-responsive cytotoxic impact on A549 and H460 lung cancer cells. The IC50 values for free GEM, free LOS, GEM@NPs, LOS@NPs, and GEM/LOS@NPs for 21.04 ± 4.15, 22.07 ± 3.69, 10.06 ± 3.91, 12.18 ± 2.84, 4.45 ± 2.57 for A549, respectively (figure 5(B)). The IC50 values for free GEM, free
LOS, GEM@NPs, LOS@NPs, and GEM/LOS@NPs for 44.79 ± 5.56, 43.05 ± 4.23, 10.03 ± 5.61, 18.79 ± 3.58, 5.4 ± 4.21 for H460, respectively (figure 5(C)). LOS and GEM were found to be about 21.04 μM and 22.07 μM for A549 cells and 44.79 μM and 43.05 μM for H460 cells, which shows the free drug and nanoformulation were induced in A549 cells compared to the A460 cells. The co-delivery of LOS and GEM was shown to be more active in arresting cell growth than the therapy of either free drug, which showed an interaction between the dual drugs that might affect in vitro cytotoxicity. Cell viability was significantly reduced when the two drugs were co-administered using PLGA-PEG NPs. Higher cellular uptake enhanced intracellular concentration and more effective anticancer drugs may have contributed to PLGA-PEG-NPs’ antitumor activity. Interestingly, the A549 and H460 could inhibit the cancer cells while treating samples. The morphological changes and ROS generation assay of A549 and H460 were performed in this present study.

3.5. Examination of morphological features
AO/EB dual labeling is another method we used to distinguish/quantify live apoptotic cells from the necrotic cells by observing nuclear alterations and apoptotic body development indicative of the apoptosis cascade [55]. Nucleic acid-sensitive cationic fluorescent dye AO is used in this study. This substance will stain both living and

Figure 3. The stability of the nanoparticles was measured by DLS measurement. (A)–(C) Changes in particle size, polydispersity index (PDI), and zeta potential of GEM@NPs, LOS@NPs, and GEM/LOS@NPs incubated in aqueous solution for 7 days. (D)–(F) GEM@NPs, LOS@NPs, and GEM/LOS@NPs incubated in DMEM cell culture medium for 7 days.

Figure 4. In vitro release of GEM and LOS from a dialysis bag containing the GEM/LOS@NPs solution at pH 7.4.
dead cells. Then increase the detection of apoptosis and discriminate among late apoptotic and dead cells. AO/EB labeling is superior to AO staining alone. Untreated A549 and H460 live cells were uniformly green (figure 6); however, in H460 cells treated with GEM@NPs, LOS@NPs, and GEM/LOS@NPs at IC50, early apoptotic cells appeared stained green and contained bright green dots in the nuclei due to chromatin condensation and nuclear fragmentation in the cells treated with IC50 showed more late-apoptotic cells and fewer necrotic ones. Only cells that have lost their membrane integrity can be stained due to the presence of EB. EB emits orange-red fluorescence when it enters a membrane-compromised nucleus. Thus, after incubation with GEM/LOS@NPs at their predicted IC50, we confirmed that apoptotic cells and normal cells could be easily discriminated by employing this AO/EB staining. It denotes apoptosis stimulation by GEM/LOS@NPs in A549 and H460.

3.6. Examination of nuclear damage
Hoechst studied GEM@NPs, LOS@NPs, and GEM/LOS@NPs-induced apoptosis necrosis by looking at the subcellular location of nucleic acids and the nucleus in cells incubated for various samples (figure 7). When attached to dsDNA, Hoechst 33342, a fluorescent DNA intercalating dye, exhibits blue fluorescence and marks apoptotic cells and nuclei. Morphologically, the apoptotic nuclei seem smaller, fragmented, and highly textured as incubation time rises. As shown in figure 7, healthy cells in the control group began to demonstrate chromatin condensations in dark blue spots and a considerable decrease in cytoplasmic size as the incubation period of GEM/LOS@NPs increased in both A549 and H460.

3.7. Examination of intracellular ROS-generation
Researchers use chemiluminescent probes and cell-permeant fluorescent probes (such as DCFFDA). We used fluorescence to monitor the redox status of A549 and H460 cells in GEM@NPs, LOS@NPs, and GEM/LOS@NPs at their IC50 concentration. DCF is formed when this non-fluorescent molecule is oxidized and becomes highly fluorescent. As a byproduct of aerobic LOS metabolism, ROS are chemically reactive species.
that are highly toxic. The term ‘oxidative stress’ refers to the state that occurs when the intracellular levels of ROS are so high that they cause considerable damage to cellular components, including lipids, proteins, and DNA. A549 and H460 cells undergo apoptosis because of mitochondrial malfunction and DNA fragmentation induced by oxidative stress. A549 and H460 cell apoptosis was evaluated by DCFH-DA assay, which measured ROS production at the IC50 concentration. The fluorescence of A549 and H460 cells was gated based on untreated control. The figure 8 shows that 97.5% of A549 and H460 control cells are healthy, whereas the apoptotic population increases dramatically when treated with their IC50 concentration.

4. Conclusion

This study used PLGA–PEG NPs to deliver LOS and GEM, two naturally occurring anticancer drugs, to lung cancer cells. It was found that the double emulsion process was used to drug-loaded NPs that were characterized using TEM and DLS studies. LOS and GEM encapsulated in PLGA–PEG NPs were shown to be more effective in inhibiting the growth of lung cancer cells than either compound alone. LOS and GEM were shown to work synergistically in inhibiting cell viability and triggering apoptosis when delivered together through PLGA–PEG NPs. Using GEM/LOS@NPs, we demonstrated an effective therapy LOS hod for lung cancer with great biomedical potential.
Data availability statement

No new data were created or analysed in this study.

Conflicts of interest

The authors declare no potential conflicts of interest.

Financial & competing interests disclosure

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