FORMULATION AND EVALUATION OF ISORHAMNETIN LOADED POLY LACTIC-CO-GLYCOLIC ACID NANOPARTICLES

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ABSTRACT

Objective: The aim of the present study was formulation and evaluation of isorhamnetin loaded poly lactic-co-glycolic acid (PLGA) polymeric nanoparticles (NPs).

Methods: The present study was designed to incorporate the isorhamnetin in PLGA formulation by double emulsion solvent evaporation method, which offers a dynamic and flexible technology for enhancing drug solubility due to their biphasic characteristic, variety in design, composition and assembly. Synthesized isorhamnetin-PLGA NPs were characterized by Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), and particle size analyzer. We tested the efficacy of isorhamnetin-PLGA NPs in HepG2 cell lines.

Results: From the FTIR result, we concluded that -C-N-, -C=C-, N-H, C-N, N-O, O-H, and C-H are the functional groups present in isorhamnetin-PLGA NPs. SEM image shows spherical shape of particles. The particle size analysis result shows 255-342 nm range of particles. Isorhamnetin-PLGA NPs significantly enhanced (p<0.05) the antiproliferative effect when compared to the plain drug.

Conclusion: This study concluded that the newly formulated NP drug delivery systems of isorhamnetin provided an insight into the therapeutic effectiveness of the designed formulation for the treatment of chemotherapy.

Keywords: Isorhamnetin, Poly lactic-co-glycolic acid, W/O/W emulsion, Antiproliferative activity, HepG2.

INTRODUCTION

The polymeric nanoparticles (NPs) are prepared from biocompatible and biodegradable polymers in size between 10 and 1000 nm where the drug is dissolved, entrapped, encapsulated, or attached to a NP surrounding substance. Depending on the way of preparation NPs, nanospheres or nanocapsules can be obtained. Nanocapsules are systems in which the drug is confined to a hollow space surrounded by a single polymer membrane, while nanospheres are matrix systems in which the drug is physically and uniformly dispersed [1-3]. Recent drug carrier systems play a key role in controlled discharge of a pharmaceutical mediator to the target at a therapeutically best possible rate and dose efficacy, reduced side effects, incessant dosing, reduced ache from administration, increased ease of use and improved mobility [4]. Drug delivery vehicles based on polymeric NPs have shown huge potential in terms of cellular uptake, overcoming drug resistance by modulation or reversion of P-glycoprotein activity, controlled drug delivery, and prolonged efficacy [5,6]. In many cases, NPs accumulate on the cell membrane and are mainly internalized by endocytosis [7].

Poly lactic-co-glycolic acid (PLGA), a Food and Drug Administration-approved biocompatible and biodegradable polymer, has been widely used to carry chemotherapy drugs, nucleic acids and proteins for cancer therapy [8-10]. PLGA NPs are colloidal polymeric drug carriers that grasp promise for peroral drug delivery which represents by far the most general and suitable way of administration. This PLGA-NPs offer a lot of advantages in excess of conventional oral dosage forms, such as enhancing the oral bioavailability of those poorly absorbed drugs, protecting the encapsulated drugs in the polymer system [11,12].

Numerous encapsulation techniques have already been developed to prepare particulate sustained drug release systems, some of the commonly reported methods of preparing NPs from biodegradable polymers include emulsion solvent evaporation [13], monomer polymerization [14], nanoprecipitation [15], cross-flow filtration [16] or emulsion-diffusion technique [17], and the salting out method. However, the selection of a particular technique of encapsulation is typically determined by the solubility characteristics of the drug [18]. At present, an enormous range of synthetic and herbal drugs, biological enzymes, minute hydrophilic and hydrophobic drugs, vaccines, and macromolecules can be loaded or encapsulated in the NPs by double emulsion solvent evaporation technique, for efficient delivery [19-22].

The flavonoid isorhamnetin also called as 3′-methoxy-3, 4′, 5, 7-tetrahydroxyflavone (Fig. 1) is the metabolite of quercetin, and it is naturally occurring O-methylated flavonol that is rich in apples, blackberries, cherries, and pears [23-25]. Recent studies have shown that isorhamnetin exerts antitumor cancer property, particularly inhibits the proliferation of numerous cancer cell lines and suppresses the weight and size of tumors of Lewis lung carcinoma cell allografts in mice [26-28].
This study provides an improved method for producing isorhamnetin loaded PLGA NPs (IR-PLGA NPs) with the following objectives: (i) Preparation of IR-PLGA NPs. (ii) Characterization of the prepared IR-PLGA NPs by FTIR, SEM, particle size analyzer (PSA), and cytotoxicity assay.

METHODS

Chemicals
Poly (DL-lactide-co-glycolide) (PLGA-50:50), with a molecular weight of 7,600-115,000, isorhamnetin (C₉H₁₂O₇) with a molecular weight of 316.26 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified eagle's medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and fetal bovine serum (FBS), were procured from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), dichloromethane and acetone, polyvinyl alcohol (PVA), PBS buffer chemicals (NaCl, KCl, Na₂HPO₄, KH₂PO₄ and HCL) were purchased from HiMedia Chemicals Pvt. Lim. Indila AB other chemicals and reagents used were of analytical grade.

Cell culture and treatment
Hepatocellular carcinoma cell line (HepG2) was obtained from National Centre for Cell Science Pune. Cells were cultured and maintained in DMEM medium, supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin (100,000 U/l penicillin, 100 mg/l streptomycin), at 37°C in a humidified atmosphere containing 5% carbon dioxide and 95% room air.

Formulation of isorhamnetin loaded PLGA NPs
Isorhamnetin loaded PLGA NPs (IR-PLGA NPs) were prepared by double emulsion solvent evaporation method [15,29]. This technique has two phases in the name of organic and in organic. In organic phase, 0.25 g of PLGA (50:50) polymer was taken in 10 ml of mixture of dichloromethane and acetone (85:15, v/v) in internal aqueous phase (IAP). 25 mg of isorhamnetin (1% dry weight of polymer) was dissolved in 40 ml of PBS (67 mM, pH 6.0). The two solutions were mixed by ultrasonication for 1 minute under cooling to form W₁/O₁ emulsion that is so called inner emulsion. The inner emulsion stabilizer was slowly added to 100 ml of 1% (w/v) aqueous PVA solution that was homogenized with a high-speed mixture for 8 min at 8500 rpm. The resulting W₁/O₁/W₂ emulsion was stirred at 300 rpm over night to maximum evaporation of organic solvent. Then, the sample was washed 3 times with Milli-Q water at 12000 rpm for 15 minutes then particles were formed. These particles were allowed to lyophilization for fine particles and preserved for further analysis.

Compatibility study - By Fourier transform infrared spectroscopy (FTIR)
FTIR
IR spectra of isorhamnetin and other excipients used in the formulation were recorded using "Perkin-Elmer FTIR." The sample for the IR spectroscopy was prepared by mixing the IR-PLGA NPs with spectroscopic grade KBr and compressed into transparent pellets, then scanned in the IR range from 500-4000/cm with a resolution of 4/cm [30].

RESULTS AND DISCUSSION

Morphology study
Scanning electron microscope (SEM)
The morphology of IR-PLGA NPs was examined by scanning electron microscopy (SEM, VEGA 3 TESCAN). The IR-PLGA NPs were mounted on metal stubs using double-sided tape and coated with a 150 Å layer of gold under vacuum. Stubs were visualized under scanning electron microscope [31].

Determination of particle size
The particle size and size distribution of the loaded PLGA (50:50) NPs were characterized by laser light scattering using particle size analyzer (Malvern Mastersizer Hydrom-2000 SM, UK). The obscuration level was set between 7 and 11%; distilled water was used as a medium.

Cytotoxicity study by MTT assay
The cytotoxicity of IR and IR-PLGA NPs was determined by the MTT assay [32,33]. Briefly, 5×10³ cells/well was plated in 96-well tissue culture plates. IR and freeze-dried IR-PLGA NPs were diluted in culture media and 100 µl of different concentrations added to wells. IR solution was replaced after every 24 hrs for 3 days while IR-PLGA NPs were added once. After 72 hrs, the supernatant was flicked off, 50 µl of MTT (0.5 mg/mL) added to each well and incubated for 4 hrs. The unreduced MTT and medium were then discarded. Each well was washed with 200 µl of PBS. 200 µl of DMSO was added to each well to dissolve the MTT formazan crystals. Plates were shaken for 20 minutes, and absorbance was measured at 540 nm using the microplate reader (Bio-Tek, ELX-800 MS) [34]. The IC₅₀ values were calculated from concentration-effect curves, considering the optical density of the control well as 100%. The experiments were repeated 5 times.

Statistical analysis
Data are represented as mean±standard deviation of four readings.

Table 1: Obtained peaks and their corresponding functional groups in IR-PLGA NPs

| Frequency (cm⁻¹) | Bond | Functional group   |
|-----------------|------|--------------------|
| 3351.54         | O-H stretch, H-bonded | Alcohols, phenols |
| 2802.00, 2383.69| C-H stretch          | Alkanes           |
| 2250.49         | C-C=O stretch        | Aldehydes         |
| 1487.41         | N-O asymmetric stretch| Nitro compounds   |
| 1449.46         | C=O stretch          | Aromatics         |
| 1300.17         | N=O symmetric stretch| Nitro compounds   |
| 1186.64, 1190.88| C=N stretch          | Aromatic aldehydes| Carboxylic acids |
| 890.17          | O-H bend             | Aldehyde          |
| 689.56          | C=O stretch          | Aldehydes         |
| 534.07          | C-Br stretch         | Alkyl halides     |

IR-PLGA: Infrared-poly lactic-co-glycolic acid, NPs: Nanoparticles
The distribution of particle size was proportioned in good conformity with the result measured by particle size analyzer (Fig. 4a and b). The particle size analysis ranges from 255 to 342 nm which shows the highest peak for IR-PLGA NPs was found to be around 300 nm. Specifically, intense peak was found at 297.4 nm. Polymeric NPs of a size around or <300 nm coated with surfactants have been proved to be able to transport drugs across the blood-brain barrier [39]. The negative electric charge attributed on the surface of NPs is due to carboxylate end groups of PLGA [40]. Many factors are known to influence the particles size, one among them is the molecular weight of polymer. However, the increase in particles size can be obtained when we increase the molecular weight (76,000-115,000 Da) of the polymer.

The promising reason could be the increase in viscosity of the polymer solution in the organic phase with increasing molecular weight poses resistance to break down the nanodroplets in a smaller size on the input of same energy [41]. Moreover, another study also stated similar results from the estradiol loaded PLGA NPs [42]. Slight increase in the encapsulation efficiency was observed with the increase in polymer molecular weight. Thus, the escape of drug molecules from denser polymer matrix becomes difficult; thereby the encapsulation efficiency increases in higher molecular weight polymer formulations [43].

Cytotoxicity studies of IR-PLGA NPs
The IR-PLGA-NPs were tested on liver cancer cells (HepG2). The cytotoxicity of IR-PLGA-NPs was significantly (p<0.05) higher (IC\textsubscript{50}=8.5 µM) than the plain drug (IC\textsubscript{50}=11.2 µM) in HepG2 cancer cells.
(Fig. 5) The IR-PLGA-NPs showed higher cytotoxicity when compared with the plain drug. The results clearly showed that the IR-PLGA-NPs had greater penetration leading to higher cytotoxic potential in cell cultures. Recently, numerous drug loaded PLGA NPs also has the tremendous cytotoxicity effects against many cancer cell lines, the prostate cancer cells LNCaP; breast cancer cells MCF-7, and MBA-MB-231 were effectively suppressed by the PLGA liposomes and NPs [44]. Similarly at this point, HeLa cells are efficiently suppressed by IR-PLGA-NPs compared with plain drug.

CONCLUSION

Conclusion drawn from the present study is that the NPs may be a suitable device for administration of isorhamnetin. From the results, -C=N-, -C=C-, N-H, C-N, N-O, O-H, and C-H are the functional groups of particles. The particle size analysis result shows 255-342 nm range of particles. Isorhamnetin in-PLGA NPs significantly enhanced (p<0.05) the antiproliferative effect when compared to the plain drug. This encapsulation method was suitable for the preparation of drug NPs and development of a system whereby drugs could be administered and maintains the energetic levels of drug for a prolonged period would be the ideal system. Hence, this study concluded that the newly formulated NP drug delivery systems of isorhamnetin provided an insight into the therapeutic effectiveness of the designed formulation for the treatment of chemotherapy.

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