

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

KANDAKUMAR SETTU, MANJU VAIYAPURI\*

Department of Biochemistry, Periyar University, Salem, Tamil Nadu, India. Email: manjubc11@gmail.com

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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.

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## 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 anticancer 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].

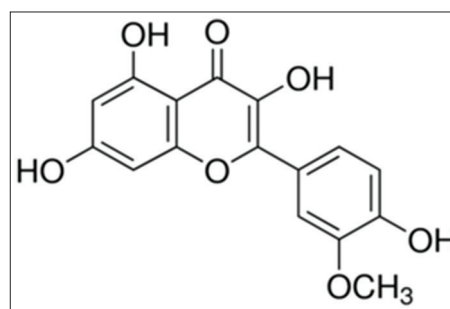


Fig. 1: Isorhamnetin

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 76,000-115,000, isorhamnetin (C<sub>16</sub>H<sub>12</sub>O<sub>7</sub>) 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, Na<sub>2</sub>HPO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>, and HCL) were purchased from HiMedia Chemicals Pvt. Lim. India. All 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 (10% 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/O emulsion that is so called inner emulsion. The inner emulsion stabilizer was slowly added to 100 ml of 1% (w/v) aqueous PVA solution which was homogenized with a high-speed mixture for 8 min at 8500 rpm. The resulting W1/O/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].

### 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 IR loaded PLGA (50:50) NPs were characterized by laser light scattering using particle size analyzer (Malvern Mastersizer Hydro-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 were determined by the MTT assay [32,33]. Briefly, 5×10<sup>3</sup> 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 IC<sub>50</sub> 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.

## RESULTS AND DISCUSSION

### FTIR

FT-IR was carried out to identify the functional groups present in IR-PLGA NPs (Fig. 2). The absorption bands at above 3351/cm may be responsible for the presence of O-H and N-H stretching vibrations. Bands at 2802/cm, 2383/cm are due to C-H stretching vibration. A band at 1584/cm showed the presence of -C=C- groups. A band at 1487/cm and 1300/cm is characteristic stretching vibration of N-O. A band at 1449/cm shows the presence of C-C stretching vibration of aromatics. Bands at 1188/cm and 1046/cm may be attributed to the presence of C-N aliphatic amines. A band at 890/cm indicates the presence of O-H bend. 689/cm and 534/cm bands showed the presence of C-Cl and C-Br stretching vibrations of alkyl halides. The obtained peak values and their respective functional groups of IR-PLGA NPs were shown in Table 1. From the FT-IR results, we concluded that -C-N-, -C=C-, N-H, C-N, N-O, O-H, C-Cl, C-Br, and C-H are the functional groups present in IR-PLGA NPs. Previously, FTIR analysis was also performed by other investigators to characterize this type of interactions between drugs and PLGA polymer [35-37].

### SEM

The obtained SEM image of the encapsulated isorhamnetin with PLGA NPs was shown in Fig. 3. The IR-PLGA NPs are well dispersed. The morphology of IR-PLGA NPs was nanometric size and spherical in shape. The surface of the particles are smooth and rounded that possesses pores of varying size. However, the rough surface of polymeric NPs could be owing to the lack of sonication during the preparation of NPs. It was reported that, if the ratio of the IAP to external aqueous phase was increased, the relative sizes of the pores also tends to increase [38].

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

Frequency (cm <sup>-1</sup> )	Bond	Functional group
3351.54	O-H stretch, H-bonded	Alcohols, phenols
2802.00, 2383.69,	C-H stretch	Alkanes
2250.49		
1584.24	-C=C- stretch	Alkenes
1487.41	N-O asymmetric stretch	Nitro compounds
1449.46	C-C stretch (in-ring)	Aromatics
1300.17	N-O symmetric stretch	Nitro compounds
1188.64, 1104.88	C-N stretch	Aliphatic amines
890.17	O-H bend	Carboxylic acids
689.56	C-Cl stretch	Alkyl halides
534.07	C-Br stretch	Alkyl halides

IR-PLGA: Infrared-poly lactic-co-glycolic acid, NPs: Nanoparticles

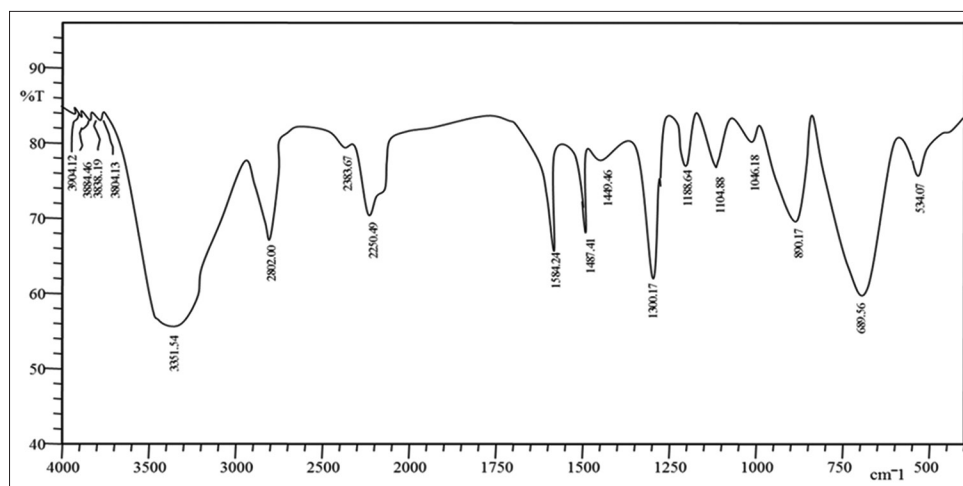


Fig. 2: Fourier transform infrared spectroscopy of infrared-poly lactic-co-glycolic acid nanoparticles

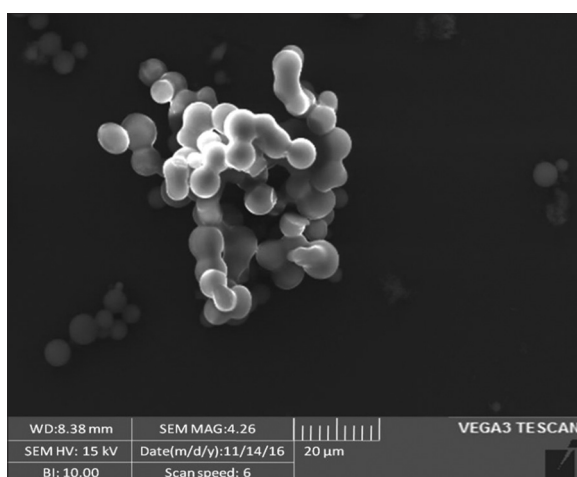


Fig. 3: Scanning electron microscopy image of infrared-poly lactic-co-glycolic acid nanoparticles

#### PSA

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].

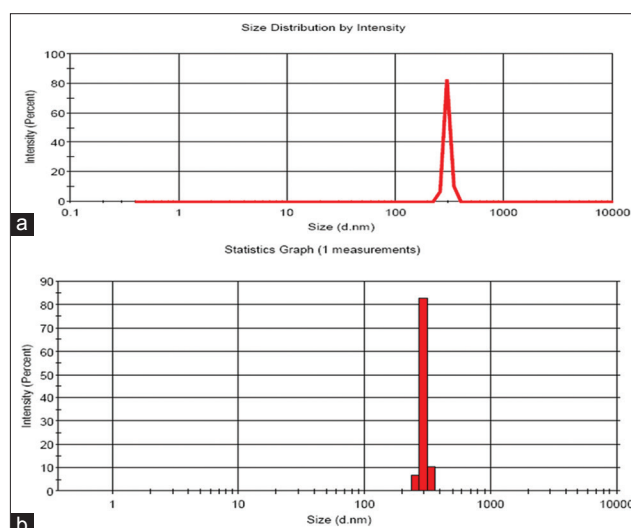


Fig. 4: (a) Particle size distribution, (b) particle size analysis by dynamic light scattering

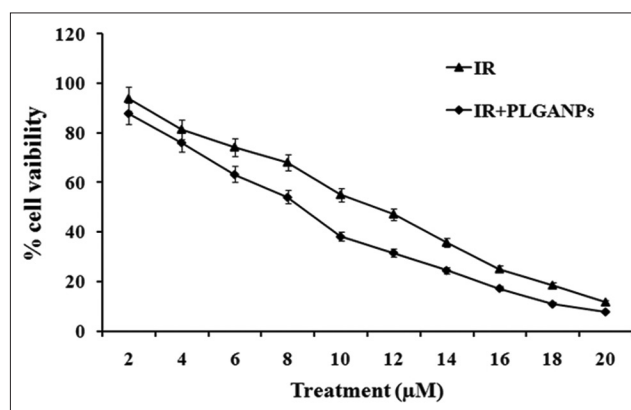


Fig. 5: Cytotoxic effect of infrared-poly lactic-co-glycolic acid-nanoparticles against HepG2 cell line

#### 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 ( $IC_{50}$ -8.5 µM) than the plain drug ( $IC_{50}$ -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 LN-CaP, breast cancer cells MCF-7, and MDA-MB-231 were effectively suppressed by the PLGA liposomes and NPs [44]. Similarly, at this point, HepG2 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 present in IR-PLGA NPs by FTIR; 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. 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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