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ORIGINAL ARTICLE

# Poly (D, L-lactide-co-glycolide)-phospholipid nanocarrier for efficient delivery of macular pigment lutein: absorption pharmacokinetics in mice and antiproliferative effect in Hep G2 cells

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## Abstract

Lutein has various biological activities, its application in food and pharma industries are limited due to poor aqueous solubility, stability, and bioavailability. To achieve various benefits, lutein-poly (lactic-co-glycolic acid) (PLGA)-phospholipid (PL) nanocapsules were prepared. Lutein-PLGA NCs (+PL) were synthesized, characterized and its bioavailability was studied in vitro and in vivo. The cellular uptake and anti-proliferative activity were analyzed in Hep G2 cells. The mean size and zeta value of lutein-PLGA NCs (+PL) were 140 ± 6 nm and − 44 mV. The amorphous nature of lutein in PLGA NCs (+PL) was confirmed by XRD and DSC. In vitro lutein release kinetics showed an initial burst followed by sustainable release up to 86%. In vitro bioavailability showed 62.7% higher lutein bioaccessibility than lutein in free form. The AUC of lutein after single oral dose of lutein-PLGA NCs (+PL) revealed 3.91-fold (plasma), 2.89-fold (liver), and 3.12-fold (eyes) higher absorption than the control (mixed micelles). The $IC_{50}$ of lutein-PLGA NCs (+PL) in Hep G2 cells at 72 h was 4.5 μM as opposed to 23.4 μM for lutein in free form. Thus, results reveal that PL added to PLGA NCs helps in enhancing the solubility which in turn resulted in its better bioavailability and bioefficacy.

**Keywords** Biomaterials · Bioavailability · Carotenoids · Hep G2 · Lutein · Nanocapsules · PLGA · Phospholipids · Stability

## Introduction

Lutein is a xanthophyll carotenoid commonly found in green leafy vegetables and fruits. Lutein gained prominence in food and pharma sector due to its wide range of biological activities like antioxidant [1], anticancer [2], anti-inflammatory [3], anti-mutagenicity [1], anti-angiogenic [4], and anti-atherosclerosis [4], and its vital role in ameliorating ocular complications like age-related macular degeneration (AMD) and cataract [5]. Being a primary antioxidant in the macula, lutein protects eyes from oxidative stress [6]. Human cannot synthesize lutein de novo and it is obtained through dietary ingestion. However, lutein bioavailability from diet is relatively low ≤ 10% due to its association with food matrices [7]. Hence, various commercial lutein formulations were emerged in the market to tackle its food security (2 to 6 mg/day). Even though studies reported on poor lutein bioavailability, wide inter-individual variation was evident due to variability in its solubility [8]. Despite of lutein's biological importance, its application in food and pharma industry is limited due to its poor aqueous solubility and stability.

In addition to poor bioavailability, lutein is sensitive to light, heat, oxygen, and intestinal pH, and its biological availability is low mainly due to the hydrophobicity of C-40 isoprenoid structure [9]. The hydrophobic nature of lutein tends to aggregate and form crystalline in aqueous solution which

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affects the intestinal absorption that in turn limits biological availability. Hence, to use lutein efficiently as an eye protective nutrient and therapeutic molecule against AMD and cancer requires strategies to protect its biological activity. Nanoencapsulation is one of the modern and novel carrier systems used to deliver and enhance the pharmacokinetics and biological activity of lipophilic molecules [10]. Earlier, we have reported that nanosized low molecular weight chitosan polymeric carriers improved lutein bioavailability in mice [11]. Even with increased biological availability of lutein from polymeric carriers, the inclusion of phospholipid (PL) to such carrier system is expected to add advantage in further improving lutein solubility, bioavailability, and bioefficacy.

Earlier, we have reported that addition of phospholipids had a positive correlation with their plasma and tissue lutein levels and helps enhanced intestinal permeation of carotenoids [12, 13]. Polymer and lipid-based carriers are two predominant delivery systems widely used and has their own advantage and disadvantages. PLGA provides stability to encapsulated lutein by protecting it from intestinal pH and other dietary factors affect lutein absorption. The phospholipid acts as an emulsifier that enhances lutein solubility, encapsulation efficiency, and biological availability. In the current study, for the first time, we have developed a polymer poly (D, L-lactide-co-glycolic acid, PLGA)-lipid (phospholecithin, PL) hybrid delivery system loaded with lutein with an aim to enhance solubility, stability, bioavailability, and its bioefficiency with combined advantage of both polymer and lipid. The PLGA-PL hybrid delivery system is expected to exhibit advantage of both carrier systems with higher lutein encapsulation efficiency, stability, and prolonged lutein release, and higher affinity towards human microfold cells which may lead to increased cellular uptake and bioefficacy and easy scale up for lutein-based food formulations.

Based on the available literature and our earlier findings, we have hypothesized that an addition of PL to polymer nanocarrier system will improve the biological availability of lutein compared to polymer based nanocarrier [14]. Further, there are no studies available on polymer-lipid nanocarrier for the lutein delivery. Hence, the aim of the study was to formulate lutein-PLGA NCs (+PL) and to characterize its bioavailability in vivo and anti-proliferative property in vitro. The outcome of the study is expected to provide better application of lutein in food and pharma industry.

## Materials and methods

### Chemicals and materials

PLGA (poly (D, L-lactide-co-glycolide) 50:50 monomer ratio, M.W (30,000-60,000), PEG (polyethylene glycol) M.W (10 kDa) and PVA (poly vinyl alcohol) were purchased from Sigma-Aldrich (St. Louis, USA.). Soya lecithin, protein-rich casein, sucrose, vitamins, minerals, cellulose, choline bicarbonate, and methionine were purchased from Hi-Media (Mumbai, India).

### Preparation of lutein-polymer-lipid nanocapsules

Marigold petals (*Tagetes erecta)* were used as a source for lutein extraction and the extract was purified and quantified by HPLC and LC-MS [11]. The self-assembled lutein-PLGA NCs (+PL) were prepared by modified nanoprecipitation method. Lutein (5 mg) and PLGA (12.5 mg) were dissolved in 2.5 mL of acetonitrile (organic phase). The lutein-PLGA NCs (+PL) was prepared by adding organic phase drop by drop into 4% aqueous ethanol solution containing PL and PEG (1:1, molar ratio) (6 mg/mL) with polyvinyl alcohol (PVA) (1%). Lutein-PLGA NCs (+PL) were formed under gentle stirring followed by high-pressure homogenization and sonication (50 Hz) (PCI Analytics, Mumbai) for 5 min. The organic phase was evaporated by continuous stirring. The lutein in free form and PVA were removed by washing NCs solution with distilled water followed by centrifugation at 12,000×*g* for 1 h at 4 °C to pellet down the NCs, freeze dried and stored in − 80 °C for further use.

### Particle size, polydispersity, and zeta potential

The particle size, polydispersity, and surface charge of NCs were analyzed by DLS zetasizer NanoZS (Malvern Instruments Ltd., Worcestershire, UK). In brief, samples were diluted with ultrapure water so that multiple scattering results from concentrated suspensions are minimized. Each sample was analyzed in triplicates at 25 °C.

### Morphology of lutein-PLGA NCs (+PL) by SEM and AFM

The surface morphology of lutein-PLGA NCs (+PL) was analyzed by SEM LEC-435 VP (LEO Electron microscopy Ltd., Cambridge, UK). In brief, a drop of sample was deposited on a carbon strip coated with gold by ion sputter allowed to dry at RT and observed under SEM. The shape and size of NCs were further characterized by Atomic Force Microscopy (AFM) (Nanosurf AG, Switzerland) equipped with Nanosurf Easyscan-2 software.

### Determination of lipid layer on the surface of lutein-PLGA NCs (+PL)

The conformation of lipid layer in lutein-PLGA NCs (+PL) was done by phase contrast microscope (Olympus, Tokyo). In brief, lutein-PLGA NCs (+PL) was stained with brilliant

cresyl blue dye at a concentration of 1 mg/mL followed by stirring and sonication. A small drop of NCs was mounted onto a glass slide with cover slip and visualized under phase contrast microscope.

## Encapsulation efficiency and lutein loading capacity

To determine the encapsulation efficiency (EE) and lutein loading capacity (LL), the nanosuspension was centrifuged at 12,000 rpm at 4 °C for 45 min. Pellet and supernatant was extracted separately and analyzed by HPLC. In brief, lutein was extracted by adding 3 mL dichloromethane: methanol (1:2, *v*/*v*) and 1.5 mL of hexane with 2 mM α-tocopherol. The above extract was vortexed and centrifuged at 1000 g for 5 min and collected the upper hexane layer. The extraction was repeated twice with 1 mL of DCM and 1.5 mL of hexane. The extracts were pooled, evaporated with a stream of nitrogen, and dissolved in a desirable volume of mobile phase (acetonitrile: DCM: Methanol, 3:1:1) and analyzed by HPLC. The EE and LL were calculated based on the following eq. (1):

$$\begin{aligned}\text{EE}\ (\%) &= \text{TL}-\text{FL}/\text{TL} \times 100; \text{LL}\ (\%)\\ &= \text{TL}-\text{FL}/\text{weight of NCs retrieved} \times 100 \qquad (1)\end{aligned}$$

where TL is the total concentration of lutein in NCs; FL is the lutein in free form available in suspension.

## XRD analysis

The physical nature of lutein in NCs was analyzed by XRD. The XRD pattern of lutein, PLGA, PL, PEG, PLGA NCs (+PL), and lutein-PLGA NCs (+PL) were recorded using X-ray diffractometer (Rigaku miniflex II desktop X-ray diffractometer, Tokyo, Japan) using Cu radiation at 30 Kv and 15 mA. The samples were examined in 2θ angle range of 6°–60° at a scanning speed of 5 °C/min with scan axis of 2θ/θ.

## Thermal characterization

In order to understand the thermal behavior of lutein, PLGA, PL, PEG, PLGA NCs (+PL), and lutein-PLGA NCs (+PL) were subjected to thermal characterization separately on double furnace Perkin Elmer DSC 8000 differential scanning calorimeter. A known quantity of sample was placed on a tin pan and the measurement was conducted at a temperature range of 10 to 150 °C with a heat flow rate of 10 °C/min under nitrogen purging.

## FT-IR spectroscopy

FT-IR (Thermo Nicolet 5700-IR: Thermo Scientific, Waltham, USA) was used to understand the interaction among lutein, PLGA, and PL. Samples were mixed with KBr to form KBr pellets using FW-4A pelletizer. Spectra were recorded in transmission mode in the region of 400–4000 $cm^{-1}$ with resolution of 4 $cm^{-1}$.

## $^{13}C$ NMR

Lutein-PLGA NCs (+PL) and PLGA NCs (+PL) samples were recorded with 500 MHz NMR spectrometer (Bruker Avans, Germany) using 32 k points, spectral width 30,000 Hz, and pulse angle of 90° with a recycling time of 2 s for $^{13}C$.

## Stability of lutein-PLGA NCs (+PL)

### Thermal and photo stability

Lutein and lutein-PLGA NCs (+PL) (10 μg/mL) were dispersed in phosphate buffer (PBS, 0.01 M, pH − 7.4) and kept in individual test tubes and collected at different time points. To find thermal stability, samples were kept in a water bath shaker (Orbitek, India) at 100 rpm, 50 °C for 48 h, and to find photo stability, samples were exposed to UV light to UV-C 254 nm TUV T5 lamp (Philips, India) for 24 h. Because of the poor solubility of lutein in PBS, 1% (*v*/*v*) Tween 80% was added. After desired time interval (4 h), samples were removed and 0.1% BHT (*w*/*v*) in ethanol was added to terminate oxidation, and samples were flushed with nitrogen and covered with aluminum foil to prevent further oxidation losses, and the lutein was extracted and quantified by HPLC.

The stability of lutein was calculated based on the following eq. (2):

$$\text{Stability of lutein}\ (\%) = C_t/C_0 \times 100 \qquad (2)$$

where $C_0$ and $C_t$ represent the concentrations of lutein at 0 h and t h, t represent duration of incubation respectively.

## Lutein release kinetics in vitro

To determine the release profile of lutein from PLGA NCs (+PL), a known concentration of lutein (1 mg/mL) was dispersed in 50 mL of freshly prepared PBS (0.01 M, pH − 7.4) and the solution was divided into microcentrifuge tubes (500 μL each). Because of the poor solubility of lutein in PBS, 1% *v*/*v* Tween 80% was added and thereafter maintained in sink conditions. The microcentrifuge tubes were incubated at 37 °C in a shaking water bath stirring at 100 rpm (Orbitek). At every 4 h time interval, the samples were drawn up to 96 h

and the tubes were centrifuged at 8000 g for 10 min to separate the released lutein from PLGA NCs (+PL). The released lutein in the supernatant was extracted and the amount of lutein released was quantified by HPLC using a calibration curve [14].

## In vitro bioaccessibility

To find out the in vitro bioaccessibility of lutein from PLGA NCs (+PL) in comparison with lutein in free form and lutein-PLGA NCs (-PL), it undergoes stimulated gastric and intestinal digestion with slight modification [15]. Lutein in free form, lutein-PLGA NCs with and without PL (200 μM), was taken in a 30-mL screw-capped glass vial and the samples were subjected to gastric and intestinal phase of digestion. Lutein in free form dispersed in medium chain triglycerides (MCT). In brief, 3 mL of pepsin (porcine gastric mucosa 88–2500 units/mg protein) in phosphate buffer (3.6 mmol/L $CaCl_2$, 1.4 mmol/L $MgCl_2.6H_2O$, 49 mmol/L NaCl, 12 mmol/L KCl, 6.4 mmol/L $KH_2PO_4$) was added to the digesta and the pH was adjusted to 2.02 with 2 mol/L HCL. The tubes were flushed with stream of nitrogen (to prevent oxidation) and were tightly capped and incubated in a shaking water bath (Scigenics Orbitek, India) for 1 h at 37 °C, 120 strokes/min (gastric phase). After incubation, the pH was elevated to 5.0 with 1 mol/L $NaHCO_3$ followed by the addition of 6 mL of 0.1 mol/L $NaHCO_3$ consist of 16 g/L pancreatin (porcine pancreas 89 U.S.P specifications) and 25.38 g/L bile extract (porcine). The pH of the digesta was further adjusted to 7.5 by 1 N NaOH and followed the above-mentioned step for sample incubation for 3 h (intestinal phase). After incubation, the aliquot of digesta (1 mL) was withdrawn from each sample at different time interval from 0 to 4 h and centrifuged (Z 360 K, BHG Hermle, Gosheim) at 12,000×*g* at 4 °C for 15 min to separate the fraction that contains micelles and this fraction was used for quantification of micellized lutein by HPLC.

## Pharmacokinetics and bioavailability of lutein in mice

To investigate the absorption pharmacokinetics of lutein from conventional micelles (control) and PLGA NCs (+PL), animal experiments were carried out after due clearance from the CFTRI animal ethics committee (IAEC NO. 396/15). Male mice [Swiss albino, IND-CFT (1c)] weighing 25 ± 2 g were fed with diet devoid of lutein for 6 weeks. The composition of the diet fed (g/kg) were casein (200), methionine (3), cellulose (50), sucrose (600), mineral mix (35), vitamin mix (10), choline bicarbonate (2), and peanut oil (100) [16]. Mixed micelles with lutein were prepared in phosphate buffered saline containing monooleoyl glycerol (2.5 mM), oleic acid (7.5 mM), sodium taurocholate (12 mM), and lutein (200 mM) [11]. The above ingredients were dissolved in chloroform separately and the solvent was evaporated to dryness using nitrogen as thin film at the sides of glass tube and the mixture was suspended in phosphate buffered saline (pH − 7.4) with vigorous mixing using a vortex mixer followed by sonication (PCI, Mumbai) for 30 min to obtain an optically clear solution, and the concentration of lutein in the mixed micelles and lutein-PLGA NCs (+PL) was confirmed by HPLC before feeding trails [17]. Devoid of lutein was confirmed by plasma lutein concentration (0.2 ± 0.01 pmol/mL) and henceforth referred as lutein devoid mice (LD mice). LD mice were divided into two groups ($n$ = 30/group). Group 1 was fed on lutein-PLGA NCs (+PL) and the group 2 was fed on micelles (control). Each group was further divided into six subgroups ($n$ = 5/time point) and assigned to different time points (2, 4, 8, 12, 24, 48 h). These groups were intubated (0.2 mL) a dose of (200 μM) either lutein-PLGA NCs (+PL) in phosphate buffer saline (pH − 7.4) (experimental group) or micellar lutein (control group). The physiological dose of 200 μM was chosen based on our previous studies that higher concentration of lutein tends to affect the bioavailability due to the saturation of lutein. At the termination of each experiment, mice were anesthetized with diethyl ether and sacrificed by exsanguinations. Blood was collected directly from the heart into heparinized tubes and centrifuged (1000×*g*, 15 min at 4 °C) to obtain plasma. Lutein was extracted from plasma, liver, and eye and analyzed by HPLC. Time for maximum absorption ($T_{max}$), absorption maximum concentration ($C_{max}$), and area under curve (AUC) were calculated.

## Cell culture and maintenance

Minimum essential medium (MEM) with Earle's salts (Himedia Pvt. Ltd., India) supplemented with 100 μg/mL streptomycin, 100 U/mL penicillin, 10% (*v*/*v*) fetal calf serum, and 2 mM L-glutamine were used for the maintenance of Hep G2 (Human hepatocyte carcinoma) cells, in a humidified atmosphere (95% air, 5% $CO_2$ at 37 °C). Lutein in free form was dissolved in known volume of DMSO and made up with fresh medium at a concentration of 0.1% (*v*/*v*). The concentration of lutein was confirmed by HPLC prior to use [18].

## Cell viability assay

Hep G2 cells were seeded at (density of $5.0 \times 10^3$ cells per well) in 96-well plate. Lutein in free form and lutein-PLGA NCs (+PL) were added to cells separately at 1, 5, 10, and 20 μM concentration and incubated at 37 °C in a humidified atmosphere with 5% $CO_2$ flow in $CO_2$ incubator (Sheldon, USA) for 24, 48, and 72 h. After incubation, 20 μL of 5 mg/mL MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) solution was added to each well and incubated for 1 h at 37 °C in a humidified atmosphere. DMSO (100 μL) was added to each well to dissolve the formazan and

it form a purple color complex. The absorbance was measured at 570 nm with a reference wavelength at 690 nm. The results are expressed as the percentage of viable cells compared to control.

$$\%\text{cell viability} = \text{mean absorbance of the control} - \text{mean absorbance of the experimental} / \text{mean absorbance of the control} \times 100 \quad (3)$$

### Cellular uptake

Hep G2 cells were seeded at (density of $5.0 \times 10^3$ cells per well) in 12-well plate. Hep G2 cells with 90% confluence were treated with lutein in free form and lutein-PLGA NCs (+PL) diluted in MEM to a final lutein concentration of 20 μM. The cells were incubated with lutein in free form, lutein PLGA NCs (+PL), and lutein PLGA NCs (-PL) at 37 °C (Sheldon, USA) for 24, 48, and 72 h. The cell monolayers were washed with precooled PBS solution for three times to stop lutein uptake. Further, 0.5 mL of 10% ethanolic PBS solution was added to dissociate cell monolayers. Cell suspensions were obtained with cell scrapers. A 0.4 mL of cell suspension was extracted and analyzed for lutein content by HPLC [12] and the remaining 0.1 mL was used for determination of protein [19].

### Statistical analysis

Data was represented as mean ± SD and tested for the homogeneity of variance by Bartlett's test. Once homogenous variance was confirmed, the data were tested by analysis of variance (ANOVA), and the significant differences in mean values among groups were analyzed by Tukey's test. The percentage difference between groups was considered significant at $p \leq 0.05$. The absorption kinetics was calculated by non-compartmental analysis using graph pad and AUC with trapezoid rule with linear interpolation.

## Results and discussion

Extracted and purified lutein (96 ± 2%) from marigold petals was quantified by HPLC and confirmed by LC-MS. Further, it was used for lutein-PLGA NCs (+PL) and mixed micelles preparation (Supplementary Fig. S-1).

### Morphology of lutein-PLGA NCs (+PL)

The surface morphology of nanoparticles plays an important role in release kinetics of drug/nutrient in vitro and in vivo [20]. The surface morphology and size of the NCs prepared in this study was measured with SEM and AFM (Fig. 1a, b). SEM micrographs of lutein-PLGA NCs (+PL) revealed that NCs were disseminated as individual particles with distinct spherical shape and smooth surface and the size distribution is less than 200 nm as compared to PLGA NCs (-PL) that are clustered in nature with spherical in shape and size distribution around 300 nm. The surface morphology and size was further confirmed by AFM. The SEM and AFM results revealed that incorporation of PL into PLGA NCs does not show much surface morphological modifications compared to NCs without PL used in our previous study [14]. The phase contrast micrograph of PLGA NCs (+PL) shows that nanocapsule formed were core-shell-shell complex. The outer layer consists of PL and PEG, the inner layer consists of PLGA, and the inner core consists of PL; lutein in the dispersed state is represented as bluish orange color (Fig. 1c, d).

### Particle size

The mean particle size (Fig. 1e) of lutein-PLGA NCs (+PL) was 140 ± 6 nm with poly dispersity index (PDI) of 0.186 which is comparatively less than PLGA NCs (-PL) as shown earlier by us as 227 ± 12 nm with PDI 0.262 [14]. The lower PDI value indicates narrow particle size distribution. The decreased particle size of PLGA NCs (+PL) compared to PLGA NCs (-PL) might be due to its higher potential to solubilize the lutein in PL [12]. The addition of PL resulted in reduced size of emulsion particles from 64.13 μm to 37.95 μm [21]. These results strongly propose the importance of PL in solubilization of lutein that results in smaller particle size. The addition of PL to PLGA NCs leads to the formation of intermolecular forces between solute and solvent and thus improves solubility of lutein in PLGA NCs. PL also retain the lutein in amorphous form with smaller particle size leads to enhanced lutein solubility and bioavailability. The particle size is one of the crucial factors which determine the cellular uptake, physical stability, and release kinetics [22]. Nanoparticles of smaller size (≤ 200 nm) are taken up and transported across the intestinal epithelial cells of GI tract more efficiently and also provide larger contact surface area for adherence to the intestine epithelial cells than the larger particles which results in higher absorption and bioavailability. Hence, the smaller the particles' size, the higher the interfacial surface of NCs which leads to higher lutein absorption and has controlled release rate than larger particle size [23].

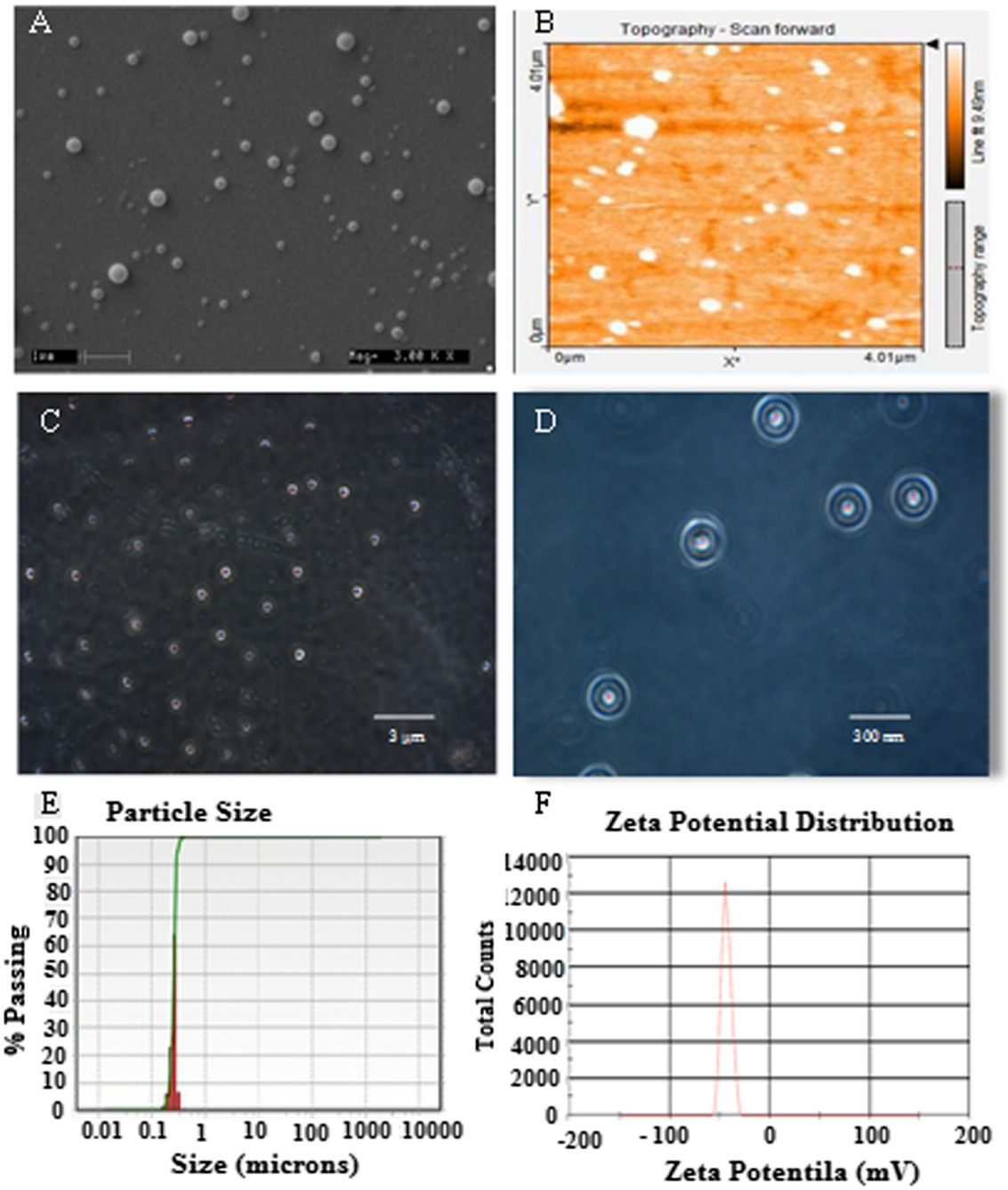


**Fig. 1** Scanning electron microscopy image, magnification × 20,000 (**a**), atomic force microscopy image (**b**), phase contrast microscopy image, magnification × 10 (**c**) and × 100 (**d**). Particle size distribution (**e**) and zeta potential (**f**) of lutein-PLGA NCs (+PL) demonstrating surface morphology, size, and surface charge

## Zeta potential

The zeta potential value (Fig. 1f) of lutein-PLGA NCs (+PL) is − 43 mV, while it was reported as − 24 mV for PLGA NCs (-PL) [14]. It is evident from the results that an addition of PL to PLGA NCs leads to increased surface charge and stability. The higher surface charge of lutein-PLGA NCs (+PL) than lutein-PLGA NCs (-PL) leads to higher particle stability [14]. The above results were supported by the addition of PL leading to increase in differential surface charge of microemulsion with and without PL as $-24.05 \pm 1.46$ mV and $7.98 \pm 2.43$ mV respectively [21]. Results indicated that PL may help in the stabilization of emulsion by increasing the surface charge and preventing particles from agglomeration [23].

## Encapsulation efficiency and lutein loading capacity

The EE and LC of lutein-PLGA NCs (+PL) were 90 ± 2% and 6.23 ± 0.03, whereas, in case of lutein-PLGA NCs (-PL), the values were 78 ± 3% and 5.15 ± 0.05 respectively [14]. The higher lutein loading efficiency compared to lutein-PLGA NCs (-PL) could be attributed to the function of PL. Results show that an addition of PL promotes EE and LC of lutein. PL act as an emulsifier and help in better solubilization of lutein which has resulted in improved EE and LC by preventing the loss of lutein into aqueous solution. [14].

## XRD analysis

The overlaid XRD pattern of lutein, PLGA, PL, PEG, PLGA NCs (+PL), and lutein-PLGA NCs (+PL), were shown in Fig. 2. The XRD was carried out to find the amorphous or crystalline nature of lutein within NCs. The diffractogram of lutein showed several well-defined peaks at a diffraction angle of 2θ range of 6–26 °C indicating high crystalline nature. The absence of lutein crystalline peak in the lutein-PLGA NCs (+PL) reveals swift in crystalline nature of lutein to amorphous or disordered crystalline phase which may be due to interaction/molecular dispersion of lutein with PLGA and PL. The amorphous or molecular dispersed phase of lutein helps in improved solubility and release kinetics of lutein from NCs which in turn enhanced the intestinal absorption of lutein. An analogous result was reported in lutein/zein nanoparticles [23].

## Thermal characterization

The physical nature of lutein within the PLGA NCs (+PL) was further confirmed by DSC. The overlaid DSC pattern of lutein, PLGA, PL, PEG, PLGA NCs (+PL), and lutein-PLGA NCs (+PL) are shown in Fig. 3. DSC peak of lutein shows a characteristic exothermic peak between 170 °C. The absence of lutein peak in PLGA NCs (+PL) indicates that lutein is packed inside capsules as amorphous or molecular dispersed nature with no characteristic lutein peak. The thermal characterization of lutein in free form is different from the nanocapsulated lutein. It is known fact that amorphous form of drug/nutrient would show enhanced solubility and bioavailability as compare to crystalline form [24].

## FT-IR

FT-IR analysis (Fig. 4) was carried to show possible interaction between lutein and other components of nanocapsules. The lutein peak at 3404 $cm^{-1}$ indicates free OH and C-H aldehyde peaks appeared at 2924 and 2953 $cm^{-1}$. Whereas, a similar C-H aldehyde peak found at 2924 and 2953 $cm^{-1}$ in lutein-PLGA NCs (+PL) indicates lutein was encapsulated inside the PLGA NCs (+PL). A sharp PLGA peak at 1728 $cm^{-1}$ indicates C=O carbonyl stretch of ester while the peak at 1086 and 1238 $cm^{-1}$ indicates C-O stretch of an ester, which are the characteristic features of PLGA polymer. The broad peak of PEG ranging from 3500 to 3300 $cm^{-1}$ is due to alcoholic (OH) group of PEG. PL characteristic ester peak is at 1728 $cm^{-1}$. A similar characteristic ester peak was also found in the PLGA NCs (+PL) with or without lutein indicating the presence of PL in both PLGA NCs. The ester C=O stretch in PLGA at 1728 $cm^{-1}$ is merged with peak appears at 1723 $cm^{-1}$ in PEG to form a broad peak as seen in both PLGA NCs (+PL) with or without lutein. When compared to the spectrum of PLGA NCs (+PL) without lutein, a difference in the spectrum at 3752 $cm^{-1}$ and 3366 $cm^{-1}$ was evident compared to lutein-PLGA NCs (+PL) indicating lutein encapsulation. These results demonstrate that there may be possible weak intermolecular forces such as hydrogen bonds between lutein and PLGA-PL.

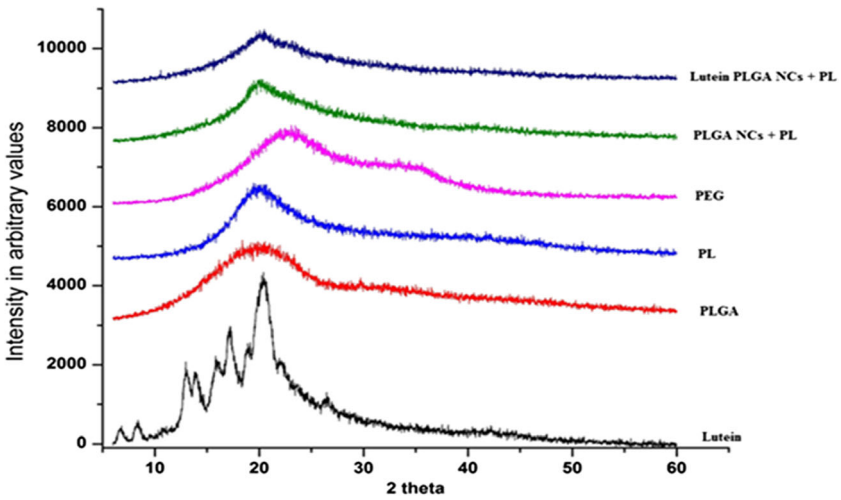


**Fig. 2** X-ray diffraction (XRD) pattern of lutein, PLGA, PL, PEG, PLGA NCs (+PL), and lutein-PLGA NCs (+PL)

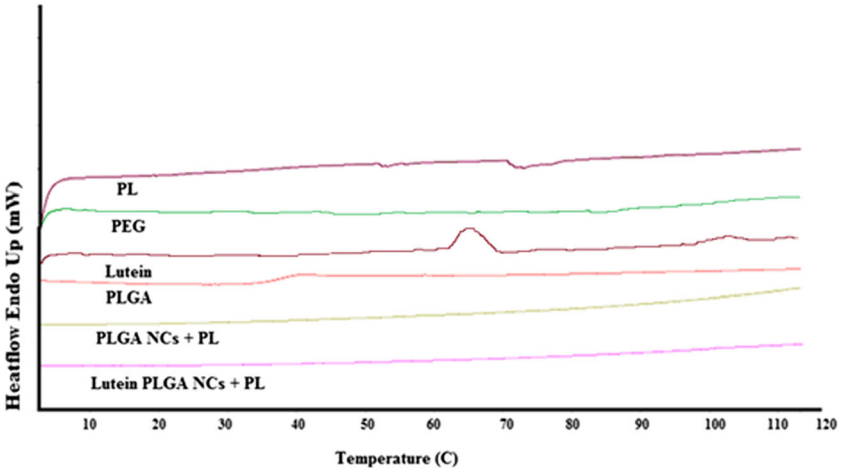


**Fig. 3** Differential scanning calorimetric of PL, PEG, lutein, PLGA, PLGA NCs (+PL), and lutein-PLGA NCs (+PL)

## $^{13}$C NMR

$^{13}$C NMR spectrum of PLGA NCs (+PL) and lutein-PLGA NCs (+PL) was shown in Fig. 5a, b. $^{13}$C NMR spectrum of PLGA NCs (+ PL) showed all the characteristic carbon signals for PLGA (16.4, 68.71, 68.86, 70.16, 166.04, 168.9 ppm), PL (13.7, 22.35, 24.71, 27.65–27.91, 28.34–29.35, 31.95, 33.78, 34.52, 72.2, 173.3 ppm), and PEG (60.4 ppm). The $^{13}$C NMR spectrum of lutein-PLGA NCs (+PL) showed characteristic carbon signals for PLGA (16.3, 68–70, 166.0, 169.0, 168.9), PL (13.7, 22.22, 22.35, 25.30, 29.35, 33.70, 33.86, 61.35, 72.2 ppm), PEG (60.4 and 60.6 ppm), and lutein (28.7–29.3 ppm). The presence of addition carbon signals at 28.7–29.3 ppm indicates the methyl carbons of the lutein. The $^{13}$C NMR of lutein-PLGA NCs (+PL) reveals that the 173 ppm of carbonyl group of PL disappears and carbonyl shift in PLGA at 168.9, 168.9 ppm and split in PEG (60.4 and 60.6 ppm compared to 60.4 ppm in PLGA NCs (+PL). The disappearance and chemical shift of certain peak may be due to weak interaction of methyl group of lutein with carbonyl group of PL. Similarly, the disappearance of carbonyl signal at 174 ppm from LMWC nanocapsules encapsulated with lutein indicates that lutein forms a hydrogen complex with LMWC nanocapsules [11].

## Stability studies

Lutein is unstable due to its isoprenoid structure with multiple conjugated double bonds [25]. Food and pharmaceutical application needs a stable lutein with no degradation. The stability of lutein in free form and PLGA NCs (+PL) was measured at elevated temperature (50 °C) for 48 h and under UV exposure for 24 h (Fig. 6a, b). Results indicated that the stability of PLGA NCs (+PL) at elevated temperature is 86% higher than the lutein in free form 4%. The stability of PLGA NCs (+PL) under UV exposure showed 75% lutein retention compared to lutein in free form 2%. Thus, the results clearly indicate that PLGA NCs (+PL) protect the lutein from external environmental factors. The stability of carotenoid depends on several factors like carotenoid type, concentration and type of polymer, lipid, surfactant composition, and contact of atmospheric oxygen. Several factors like interfacial layer composition, pH,

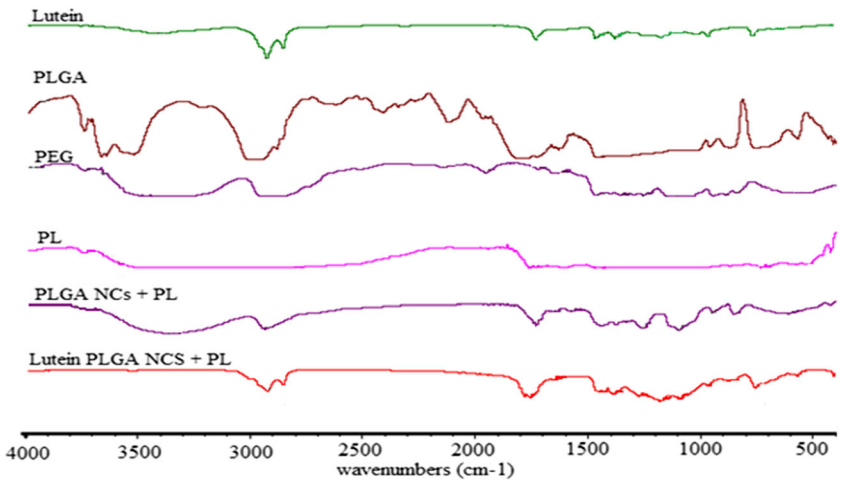


**Fig. 4** Fourier transform infrared chromatograph (FT-IR) of lutein, PLGA, PL, PEG, PLGA NCs (+PL), and lutein-PLGA NCs (+PL)

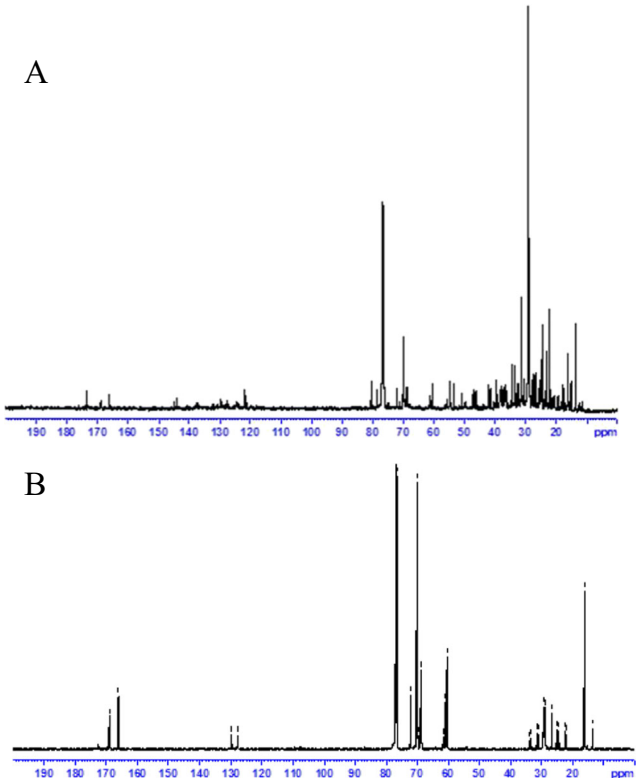


**Fig. 5** $^{13}C$ NMR of PLGA NCs (+PL) (**a**) and lutein-PLGA NCs (+PL) (**b**)

light, oxygen, and temperature affects the stability of lutein [26]. In this study, we have taken care to prevent degradation of lutein by other external factors.

## In vitro lutein release

The in vitro release kinetics of lutein from PLGA NCs (+PL) in physiological condition at 37 °C (Fig. 6c) was found to occur in two phases with initial burst release and slow sustainable release. An initial burst release of lutein (19%) was evident over 4 h followed by a controlled sustainable release (86%) up to 96 h. Whereas, in the case of PLGA NCs (-PL), the initial burst release was 16% up to 4 h and controlled sustainable release of 74% up to 96 h [14]. The increased initial burst release of lutein from PLGA NCs (+PL) compared to PLGA NCs (-PL) indicates enhanced lutein encapsulation efficiency which may be due to the inclusion of PL in the nanopreparation. The controlled sustainable lutein release may be due to the slow lutein release from NCs of the lipid core surrounded by polymer matrix. Further, the higher lutein release was observed from PLGA NCs (+PL) compared to PLGA NCs (-PL) [14]. This may be due to higher molecular dispersibility and encapsulation efficiency of lutein.

It is evident from the literature that lipid-based emulsions lack controlled sustainable release for a desired period of time. Lutein release pattern from lipid nanoemulsion was at triphase with 20% lutein release in 24 h [27]. The lutein release in vitro from self-assembled phospholipids suspension was reported to be 80.72% in 30 min [28]. These studies show that the addition of PL to PLGA-PEG NCs helps in prolonged release of lutein. The sustainable release characteristic of lutein from PLGA NCs (+PL) would have an advantage in improved bioavailability of lutein and its effect as an antioxidant

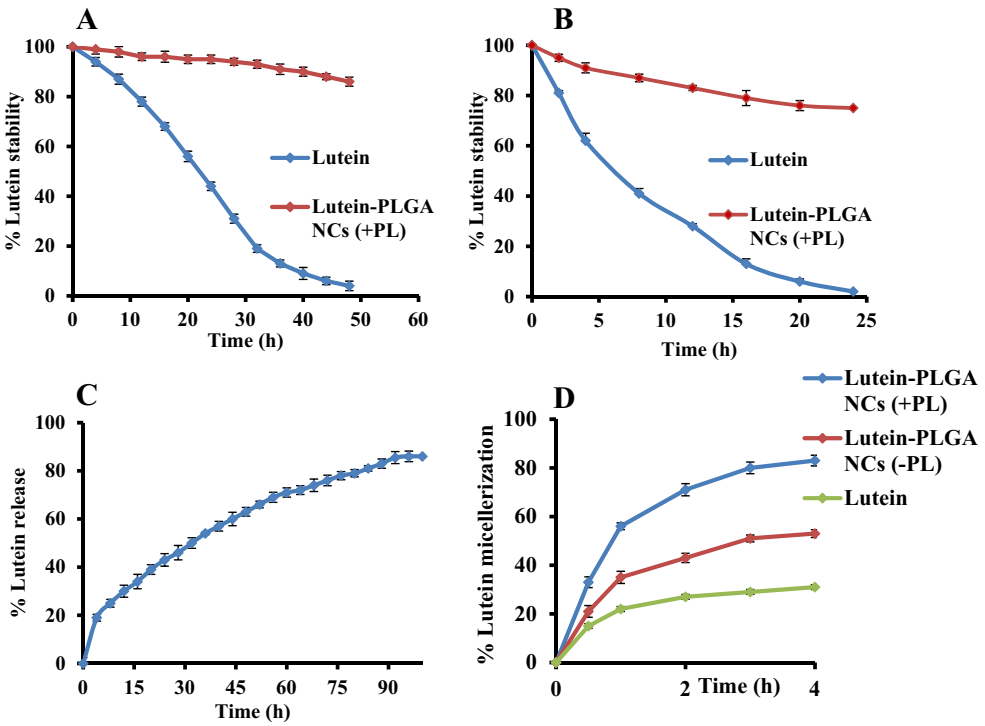


**Fig. 6** In vitro stability of lutein in free form and lutein-PLGA NCs (+PL) in physiological condition at 50 °C (**a**) and under UV exposure for 24 h (**b**), values are mean ± SD ($n = 5$). In vitro release kinetics of lutein from lutein-PLGA NCs (+PL) in PBS (pH − 7.4) (**c**), values are mean ± SD ($n = 3$). In vitro digestion showing the lutein-PLGA NCs (+PL) resulted in higher percent micellization than lutein-PLGA NCs (-PL) and control (51%) (**d**)

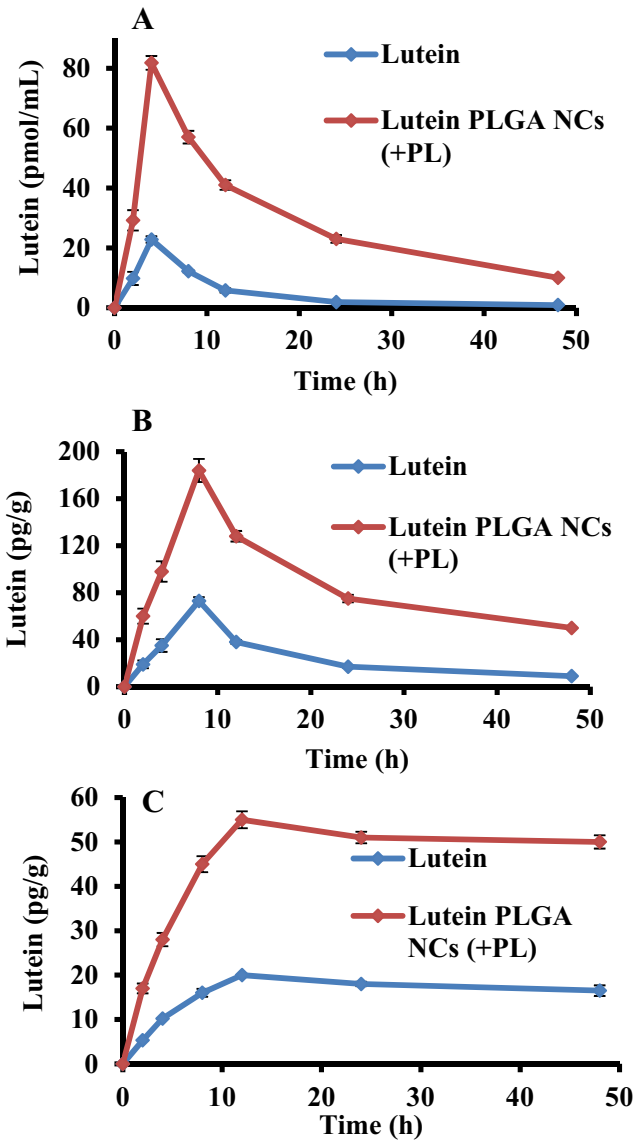


**Fig. 7** Pharmacokinetics of plasma, liver, and eye response of lutein from mixed micelles (control) and lutein-PLGA NCs (+PL), data represents the mean ± SD, ($n = 5$)

in cancer treatment and as macular pigment to safe guard visual function of AMD subjects. There are certain factors like biomaterial type, size, system, architecture, biodegradability mechanism, and drug and carrier interaction that will influence the drug release [29]. It is necessary to select a better delivery system such as polymer-lipid nanocarrier which helps in prolonged sustainable release with longer blood circulation time to obtain maximum therapeutic effect. The above results also demonstrate that the role of PLGA and PEG in controlled sustainable lutein release for a longer duration and addition of PL to PLGA NCs will be a better delivery system with added advantage of polymer and lipid nanocarrier for lutein.

## In vitro bioaccessibility

In vitro bioaccessibility studies (stimulated gastric and intestinal digestion) revealed that the percentage micellization of lutein (i.e., available for absorption by the intestinal mucosa) after 4 h of digestion shows PLGA NCs (+PL) was significantly higher than PLGA NCs (-PL) (35.0%) and lutein in free form (62.7%) (Fig. 6d). The bioaccessible lutein obtained from different time points shows that lutein bioaccessibility increased proportionally with incubation period. However after 3 h of digestion, there was not much increase in bioaccessible lutein. This result clearly demonstrates that addition of PL to PLGA NCs helps in more bioaccessibility of lutein. The addition of PEG and PLGA also helps in higher lutein bioaccessibility by protecting lutein against acidic gastrointestinal conditions [30]. The lutein in free form dispersed in MCT shows lower micellization due to possible degradation in acidic pH. Micellization of nutrients is an essential step in the process of food/drug digestion before cellular uptake [31]. Measuring bioaccessible lutein for intestinal uptake is one of the important criteria in bioavailability. Earlier reports demonstrated that addition of PL as lutein carrier improved micellization of lutein in vitro [32, 13, 17].

## Pharmacokinetics study in mice

The postprandial plasma, liver, and eye lutein response after a single pharmacological oral dose of micellar (control) and PLGA NCs (+PL) lutein in mice are shown in Fig. 7. The $C_{max}$ and AUC of plasma lutein from PLGA NCs (+PL) showed that the lutein level was higher by 3.58- and 3.91-fold respectively compared to the control group. Similarly, liver $C_{max}$ and AUC of lutein-PLGA NCs (+PL) showed that the lutein bioavailability was higher by 2.52- and 2.89-fold higher and eye $C_{max}$ and AUC showed 3.36- and 3.12-fold higher than the control group (Table 1).

The improved lutein bioavailability from lutein-PLGA NCs (+PL) may be due to enhanced solubility, intestinal permeability, better stability of nanocapsulated lutein against gastric intestinal pH, prolonged sustainable and controlled release of lutein, longer circulation of lutein in blood, and minimal interaction of nanocapsulated lutein with other food matrixes, which may cause a negative effect on lutein bioavailability. Additionally, the amorphous nature and smaller particle size nature of lutein nanocapsules (Fig. 2a) may render easy intestinal permeation compared to the micron-sized mixed micelles (12–43 μm) [14]. Further, the choice of lipid and its fatty acid composition also plays a vital role in carotenoid bioavailability. The PL used in this study as lipid source has two long chain acyl moieties and rich in oleic acid (18:1) that help in better intestinal absorption of lutein [13]. In the intestine, PL is hydrolyzed to lyso-phosphatidylcholine by phospholipase reported to aid the emulsifying process of lutein into a soluble

**Table 1** Pharmacokinetics parameters of plasma, liver, and eye response of micellar lutein and lutein from PLGA NCs (+PL) after a single oral dose to mice previously fed on diet devoid of lutein (LD mice)

| Parameters | Plasma | | Liver | | Eye | |
|---|---|---|---|---|---|---|
| | Lutein micelles (control) | Lutein-PLGA NCs (+PL) | Lutein micelles (control) | Lutein-PLGA NCs (+PL) | Lutein micelles (control) | Lutein-PLGA NCs (+PL) |
| $T_{max}$ (h) | 4 | 4 | 8 | 8 | 12 | 12 |
| $C_{Max}$ (pmol/mL or g) | 22.8 ± 1.3 | 81.8 ± 5.0* | 73 ± 1.3 | 184 ± 5.1* | 19.8 ± 1.3 | 66.6 ± 5.1* |
| $C_{last}$ (pmol/mL or g) | 0.9 ± 0.04 | 10.6 ± 0.3* | 9.3 ± 0.4 | 50.47 ± 0.8* | 1.3 ± 0.04 | 11.0 ± 0.3* |
| $C_0$ (pmol/mL or g) | 0.21 ± 0.03 | 0.22 ± 0.01 | 0.41 ± 0.02 | 0.43 ± 0.03 | 0.29 ± 0.03 | 0.30 ± 0.02 |
| AUC (p mol/mL/48 h or g) | 181 ± 17 | 709 ± 12* | 858 ± 12 | 2457 ± 19* | 164 ± 10 | 513 ± 12* |

*$P < 0.05$

form with no precipitation [13]. The intestinal permeability of lutein from nanoemulsion with PL in human subjects for 1 week showed 28 and 31% higher serum lutein level compared to commercial lutein supplement pills [33]. Bioavailability of lutein from self-emulsifying microemulsion with PL in rats reported 4- to 11-fold higher plasma lutein concentration compared to no PL emulsion [28]. These findings demonstrate that presence of PL in lutein-PLGA NCs can be potential lutein carrier to achieve enhanced bioavailability. Based on the results obtained, we propose that lutein delivery in the form of PLGA NCs (+PL) could be one of the modes of delivery for its higher bioavailability. The addition of PL to the carrier system added an advantage in the absence of bile. Absence or inadequate secretion of bile due to health complications leads to poor absorption of lutein that was ruled out by the addition of PL.

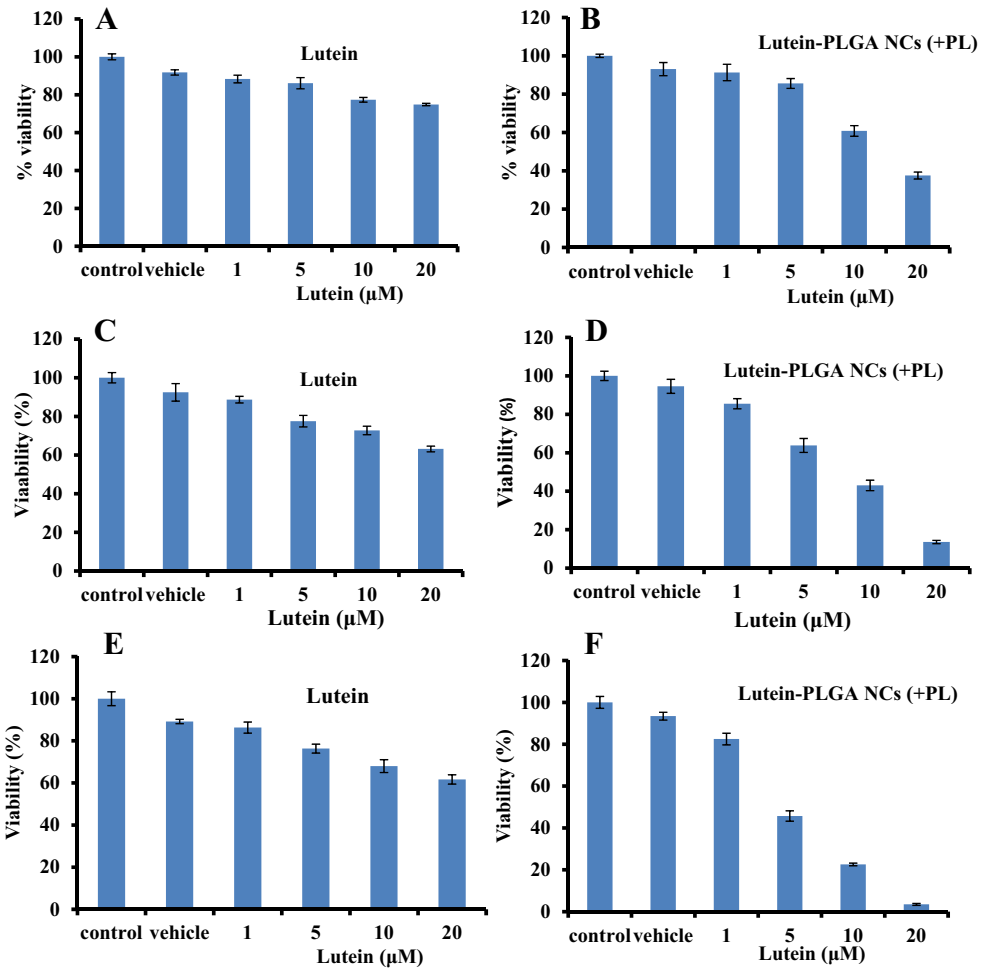


**Fig. 8** Percent cell viability of Hep G2 cells treated with 1 to 20 μM concentrations of lutein/lutein-PLGA NCs (+PL) for 24 h (**a**, **b**), 48 h (**c**, **d**), and 72 h (**e**, **f**)

However, the mode of absorption of lutein from NCs is either through receptor-mediated transport (active absorption) or through simple membrane diffusion (passive absorption). The intestinal M cells may be a more efficient cellular route for PLGA nanoparticle absorption compared to the micelles since they lack microvilli but possess broader microfold cells to transport antigens via endocytosis [34]. Hence, it requires further investigation.

## Anti-proliferative activity

In order to find out the anti-proliferative effect of lutein-PLGA NCs (+PL) (1, 5, 10, 20 μM) in comparison with lutein in free form in Hep G2 cells, cell viability was tested by treating either lutein in free form or lutein-PLGA NCs (+PL) for up to 72 h. In lutein-PLGA NCs (+PL), Hep G2 cells displayed reduced cell viability in dose-dependent manner (Fig. 8). (Supplementary Fig. S-2). However, upon treatment with lutein in free form (20 μM, the highest concentration used), the cell viability was 74%, 63%, and 61% after 24, 48, and 72 h, respectively compared to control. In contrast, treatment of lutein-PLGA NCs (+PL) (20 μM, calculated based on the percent encapsulation efficiency, the cell viability was reduced by 37%, 1%, and 4%, respectively during 24, 48, and 72 h compared to control. The 50% inhibitory concentration $IC_{50}$ of lutein-PLGA NCs (+PL) at 72 h was less than 4.5 μM as opposed to 23.4 μM for lutein in free form. The anti-proliferative effect of lutein-PLGA NCs (+PL) was more effective than the lutein in free form and lutein-PLGA NCs (-PL) ($IC_{50}$, 10.9 μM) [14].

The $IC_{50}$ of polymer-lipid-nanovesicles (LN-CPTX) loaded with paclitaxel compared with free paclitaxel in HeLa (human cervical cancer cells) was 170.8 ± 48.6 nM and 299.4 ± 42.7 nM respectively, and in B16F10 (murine melanoma cells) was 84.3 ± 3.4 nM and 157.3 ± 15.3 nM respectively. These results clearly show that minimum drug concentration is sufficient to attain the required therapeutic effect and nano-formulation shows higher cellular uptake and cell viability over free paclitaxel [31]. This higher anti-proliferative activity of lutein-PLGA NCs (+PL) may be due to the higher lutein uptake, prolonged activity, and stability of lutein nanocapsules in cellular media.

## Cellular uptake studies

The lutein uptake from lutein-PLGA NCs (+PL) in Hep G2 cells after 24 h showed 34.6% and 589% higher compared to lutein-PLGA NCs (-PL) and lutein in free form respectively. Similarly, lutein-PLGA NCs (+PL) showed higher uptake (31% and 57%) at 48 h and (28% and 60%) at 72 h compared to lutein-PLGA NCs (-PL) and lutein in free form (Fig. 9). The higher cellular uptake of lutein from lutein-PLGA NCs was due to nanosize and addition of PL which helps in improved

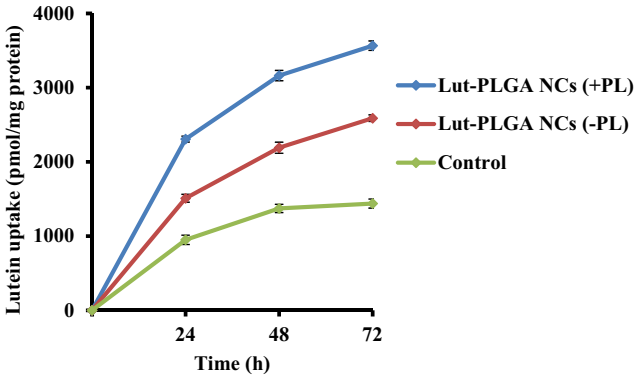


**Fig. 9** Uptake of lutein by Hep G2 cells monolayers incubated with lutein in free form dispersed in DMSO, lutein-PLGA NCs (+PL), and lutein-PLGA NCs (-PL) at a lutein concentration of 20 μM. Values were expressed as mean ± STD ($n = 3$)

solubilization and absorption of lutein. The role of PL in improved absorption is clearly demonstrated by difference in lutein uptake from lutein-PLGA NCs (+PL) compared to lutein-PLGA NCs (-PL). Lutein in free form forms crystalline in cell culture media, whereas PLGA NCs was amorphous in nature. The cellular uptake of lutein-PLGA NCs was significantly different ($p < 0.05$) from other groups.

Thus, the higher activity of lutein-PLGA NCs (+PL) compared to lutein in free form may be due to the following reasons: improved cellular uptake of lutein-PLGA NCs (+PL) than lutein in free form, since lutein-PLGA NCs (+PL) may interrelate well with the cellular membrane by virtue of both polar and non-polar groups than lutein in free form which is hydrophobic in nature. Lutein is secured in PLGA NCs (+PL) in the cellular environment for longer time, where degradation of lutein is minimum compared to lutein in free form. The constant release of lutein from lutein-PLGA NCs (+PL) resulted in continuous supply (controlled release) and accumulation of lutein at tumor sites which can maintain the activity for a longer period as opposed to the lutein in free form. The Hep G2 cells treated with PLGA NCs (+PL) showed no anti-proliferative effect as it emphasize that the anti-proliferative activity is due to lutein but by PLGA NCs (+PL). Similarly, nanoencapsulated curcumin exhibited lower ($IC_{50}$) (31 μM) value compared to free curcumin (37 μM) in LNCaP, $PC_3$, and DU 145 prostate cancer cells as it indicates nanocurcumin formulation is more effective than the free curcumin in treating prostate cancer [20]. From our present results, it is evident that PLGA NCs (+PL) may be used as a better therapeutic carrier for lutein than the lutein in free form to treat cancer cells.

# Conclusions

The nanoencapsulation of lutein in PLGA NCs (+PL) leads to improved lutein solubility which in turn enhanced the

intestinal uptake, plasma, and tissue response of lutein. However, the molecular mechanism of intestinal uptake of lutein from PLGA NCs needs further investigation. The higher lutein absorption from PLGA NCs (+PL) may be due to the nanosize carrier (≤ 200 nm) in which lutein is loaded, higher EE, better solubilization, stabilization, and self-emulsification property of PL added to PLGA NCs. Results demonstrate that PLGA-PL hybrid nanocapsules can serve as efficient delivery system than polymer or lipid delivery system which combines the advantages of both polymer and lipid nanocarrier for improved solubility, stability, bioavailability, and anti-proliferative activity of lutein. These findings help in better application of lutein in food and pharma industry to meet the lutein security to mitigate cancer and AMD complications.

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## Compliance with ethical standards

**Ethical standards** Manuscripts submitted for publication must contain a declaration that the experiments comply with the current laws of the country in which they were performed.

**Animal studies** All institutional and national guidelines for the care and use of laboratory animals were followed.

**Conflict of interest** The authors declare that they have no conflict of interest.

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