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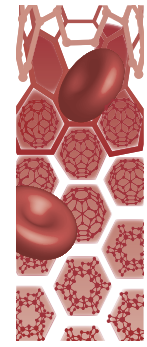

pH-sensitive nanoparticles containing 5-fluorouracil and leucovorin as an improved anti-cancer option for colon cancer

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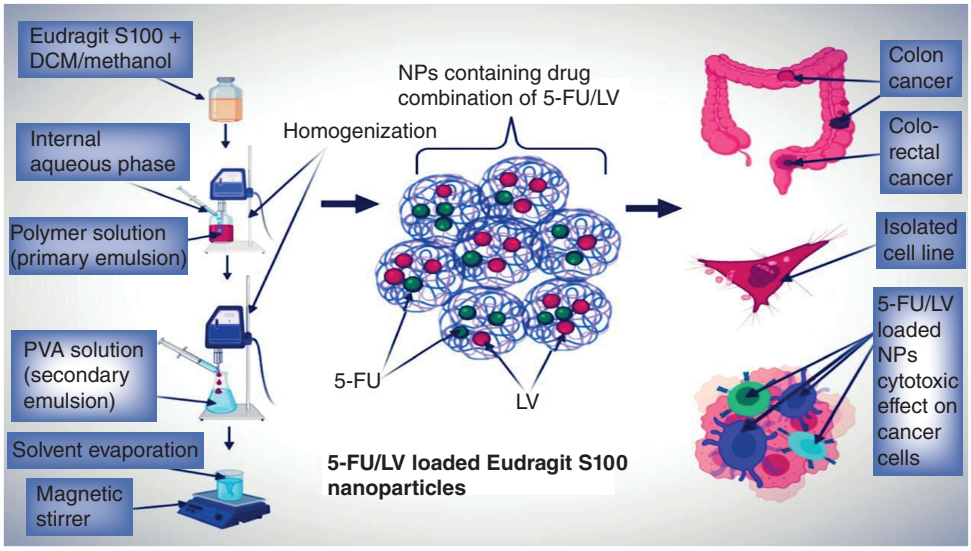
Background: Parenteral administration of chemotherapeutic drugs, 5-fluorouracil (5-FU) and leucovorin (LV), is commonly used to treat large bowel carcinomas such as colon cancer (CC) and colorectal carcinoma (CRC). **Aim:** Our study aims to design a novel nanoparticulate drug-delivery vehicle for oral use capable of colon-specific release. **Methods:** A modified double-emulsion solvent evaporation method was used in the preparation of pH-responsive Eudargit® S100 polymeric nanoparticles, loaded with 5-FU/LV combination (5-FU/LV-loaded Eudargit S100 NPs). **Results:** Our optimized drug-loaded NP showed a pH-responsive drug release and exhibited significantly more cytotoxic actions in cancer-cell lines than free drugs. **Conclusion:** These findings open the way for conducting clinical trials for colon malignancies treated with nanoparticles.

Graphical abstract:



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Keywords: 5-fluorouracil • colon targeting • eudragit S100 • large bowel carcinoma • leucovorin • nanoparticles • oral delivery

For decades, researchers have struggled to discover and foster appropriate treatment methodologies for different cancers [1–3]. Colorectal carcinoma (CRC) was ranked as the fourth commonly diagnosed cancer type that showed a high morbidity rate of about 1 million global deaths in the previous decade and was ranked as the third top mortality-related malignancy worldwide [1–3]. The pathogenesis of CRC remains increasingly complex and interwoven.

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Colorectal carcinoma (CRC) first affects the lining mucosa of colon areas and later progresses to serious rectum-related symptoms, including bleeding, diarrhea, abdominal pain, abscesses, fistulas and constipation. Finally, it affects the dietary habits [4–8].

The colon drug-delivery systems (CDDS) should be formulated considering four major approaches such as (i) pH-dependent CDDS (polymer-based nanosystems, lipid-based formulations and film-coated tablets or capsules); (ii) enzyme-sensitive CDDS (polysaccharide-based systems); (iii) ligand/receptor-mediated CDDS (antibodies, peptides); (iv) magnetically driven CDDS. However, each approach has its own advantages and disadvantages [8]. Colon disease treatment using nanoparticulate drug-delivery systems is considered as a promising therapeutic modality in CDDS [4,9,10].

The oral drug-delivery route is the most common route for treating colon diseases [6,7,9,11–15]. Paul Ehrlich's 'Drug targeting' approach is focused on delivering therapeutic drug active components directly to cancer cells, ensuring maximum efficacy with minimal adverse effects [16–18]. Polymers should avoid hydrolysis in the upper part of the gastrointestinal tract (GIT) to ensure colon targeting success and protect drugs against destructive GIT conditions [16,19]. Unfortunately, transit time may affect drug release because of the oral form's inability to disintegrate in the colon [9,13,20–22]. Nowadays, aspiring efforts tend to design innovative drug nanocarriers that can reach the site of action to deliver the optimum drug dose with minimal adverse effects [10,16,23].

Nanotechnology has a significant role in the treatment of CRC. Multiple unit systems such as microsphere (MS) and nanosphere (NS) formulations are promising techniques for colonic drug targeting [13,24–27]. The pH threshold and colon solubility should control the polymer selection in the case of pH-dependent CDDS [25,28]. The micro/nanoencapsulation method should be selected according to the drug and polymer's unique physicochemical properties [17,29–31]. Therefore, the designed nanocarrier advocated for the treatment of CRC can protect the loaded therapeutics against intestinal surroundings and maintain drug release in colon areas, increasing colon targeting and therapeutic efficacy [10].

Colon targeting using pH-sensitive polymers of methyl methacrylic acid (Eudragit® S100) with a dissolution starting at pH of 7–7.4 similar to the colon pH is a reliable targeting technique. Using these polymers has many advantages such as less pH effect and lower dose dumping risk, enhancement of uptake, and bioavailability of acid-labile and hardly soluble drugs. Eudragit S100 inhibits the drug releases in the terminal ileum and increases colonic selectivity for drug release [5,20].

For more than 50 years, 5-fluorouracil (5-FU; a type of fluoropyrimidine) was used as the first choice for the treatment of various tumors [32–36], including colonic, head, breast and neck cancers. 5-FU has adverse effects and low bioavailability, and 5-FU has a 10% response rate [37] on cancerous cells in addition to a short half-life and variable oral bioavailability. The combination of oral 5-FU and leucovorin (LV) is used for stage two or three colonic cancer patients with equivalent efficacy for intravenous combined therapy [25,38]. The therapeutic response of 5-FU against CC and CRC will be readily enhanced by adding another biochemical modulator such as LV [25,37,39] especially in metastatic CRC patients [1,11,21,40]. The oral combination of LV and 5-FU shows successful treatment in advanced CRC patients due to the high potential of LV to boost 5-FU antitumor activity by augmenting the inhibitory effect to thymidylate synthase, which is the key enzyme responsible for tumor DNA and RNA synthesis [25,41–43].

The 5-FU/LV-loaded Eudragit S100 NP administration can extend the therapeutic efficacy against colorectal cancer by exploiting synergistic drugs combination [26]. Folate-decorated chitosan nanoparticles had been applied as a delivery carrier for 5-FU/LV by ionic gelation depending on the aqueous solubility of both drugs and polymer [44]. Both drugs of hydrophilic nature provide high encapsulation efficacy [32] within the polymer matrix. 5-FU/LV-loaded chitosan NPs exhibited selective targeting of both medications to CRC. The 5-FU-loaded SLN prepared by the temperature-modulation solidifying method shows a higher uptake and retention inside the colon cancer cells [42]. Both PLGA and Eudragit S100 can be used by the emulsion-droplet coalescence method to deliver 5-FU to the cancerous colon tissue due to the pH-sensitivity of Eudragit S100 and biocompatible and biodegradable properties [4,29,45–47] of the PLGA.

This study aims to prepare 5-FU/LV-loaded Eudragit S100 nanoparticles using the double emulsion solvent evaporation method and to optimize the preparation of 5-FU/LV-loaded Eudragit S NPs using different formulation variables such as PVA concentration, amount of polymer and organic solvent to external phase volume ratio (O/W2), which can affect the physical and chemical features of the prepared nanoparticles. Furthermore, this study is focused on investigating the optimized 5-FU/LV-loaded Eudragit S100 cytotoxic action NPs on the CC and CRC cell lines *in vitro*.

Materials & methods

Eudragit S100 was received as a gift from Evonik Nutrition & Care GmbH (Berlin,Germany). 5-fluorouracil (5-FU), folinic acid (leucovorin), poly(vinyl alcohol) (PVA; 87–89% hydrolyzable; Mw~30,000–50,000) & dialysis tubing cellulose membrane 25 mm with M.wt. cut-off 12,000 Da, respectively, were purchased from Sigma-Aldrich chemical co. (MO, USA). Dichloromethane (DCM), methanol, sucrose, sodium dihydrogen phosphate and hydrochloric acid (HCl) were obtained from El-Gomhoria co. for chemical industries (Mina, Egypt). Milli-Q® water, and all solvents were of analytical grade.

Preparation of 5-FU/LV-loaded NPs

In this work, a modified double emulsion (W1/O/W2) solvent evaporation method was utilized. The internal aqueous phase containing a mixture of both drugs (5 mg of 5-FU and 2 mg of LV) was mixed with an organic phase of dichloromethane (DCM) and methanol containing different Eudragit S100 concentrations. An ultrasonic homogenizer emulsified the two phases with a 3.2 mm probe sonicator (Cole-Parmer 4710 series, AK, USA) to prepare the first W1/O emulsion. Subsequently, the primary emulsion was injected into polyvinyl alcohol (PVA) solution for 5 min to get the secondary emulsion. To remove the excess organic solvent, the double emulsion system was stirred using a magnetic stirrer (Janke & Kunkel GmbH & Co. KG, Staufen, Germany) for 3 h under a vacuum. The whole system was centrifuged at 25,000× *g* for about 30 mins at 5°C using (Sigma Laborzentrifugen GmbH., Berlin, Germany), and the supernatant containing drug-loaded nanoparticles was washed three times with Milli-Q (H_2O) and sucrose solution at 2% (w/v) then freeze-dried, by freeze-dryer (Labconco., MO, USA). Finally, the resulting NPs were stored at room temperature in a desiccator [32,48].

Physicochemical properties of 5-FU/LV loaded nanoparticles

Particle size & polydispersity index

Loaded NPs size and polydispersity index (PDI) were determined by photon correlation using dynamic light scattering (Zetasizer 5000, Malvern Instruments, Worcestershire, UK). Lyophilized NP samples were diluted using Milli Q water. Measurement of each sample was repeated three times [49,50].

Zeta potential

The analysis of zeta potential was carried on freeze-dried samples using laser dropper electrophoresis (Zetasizer 5000, Malvern Instruments, Worcestershire, UK). Nanoparticle suspensions were diluted by using KCl solution (0.001 M) to adjust its conductivity, and the average of three times consecutive measurements was calculated [51].

Morphology of 5-FU/LV-loaded NPs

The morphological examination of the 5-FU/LV-loaded NPs was carried out using scanning electron microscopy (SEM) (FEI Quanta 400 FEG, FEI). An aliquot of NP was mounted on carbon tape and sputter-coated with gold under vacuum before SEM imaging [52].

Determination of 5-FU/LV encapsulation efficiency

To detect 5-FU and LV content, an indirect approach was used to determine 5-FU and LV amounts in the supernatant after nanoparticle suspension centrifugation. Both drugs concentrations were determined by first derivative UV spectroscopy using double-beam UV-spectrophotometer (ThermoScientific™ Evolution™ 350 UV-Vis Spectrophotometer). The wavelengths used for UV detection were λ_{max} = 265 nm and 286 nm for 5-FU and LV, respectively. The variation between the original 5-FU, LV amounts added, and the un-entrapped drugs still in the supernatant after NP manufacture was used to compute 5-FU and LV encapsulation in the NPs. The mean encapsulation efficiency (%EE) of 5-FU and LV was computed for every sample in triplicate [18].

The following equation was used to estimate the drug encapsulation efficiency (%):

$$\%EE = \frac{I - S}{I} \times 100\%$$

where S is the drug amount that remained in the supernatant and I is the initial drug amount.

In vitro release studies

Certain nanoparticle suspension volume was placed in a dialysis bag (MWCO 12,000 Da). The tight tubed bag was submerged in 50 ml of aqueous receiver phase of four different pHs (0.1 N hydrochloric acid of pH = 1.2 and phosphate buffers of pH = 4.5, 6.8, and 7.4 for simulated gastric fluid (SGF) and simulated intestinal fluids (SIF)), respectively [53] which depends on the gradual pH change in the digestive tract that varies between the stomach (pH 1.2) and the colon (pH 7.4) [54]. The incubation times in various gastrointestinal simulated release mediums were as follows: the first 2 h at pH of 1.2, the 2–4 h at pH of 4.5, the 4–6 h at pH of 6.8, and the 6–8 h at pH of 7.4 [55]. Agitation was done by magnetic stirrer at 50 r.p.m. at ~37°C ± 1 under sink conditions. An aliquot of receiver phase (2 ml) was removed at time intervals of (15, 30, 45, 60, 90, 120, 150, 180, 240, 300, 360, 420, 480, 540 and 600 min and analyzed using a UV-Visible spectrophotometer and substituted with an equal volume of fresh buffer medium [56,57].

Culture of CT26 & HT-29 cancer cell lines

Murine colon carcinoma (CT26) cells were incubated in RPMI complete medium supplemented with FBS (10% v/v), L-glutamine (2 mmol l^{-1}), penicillin (100 U ml^{-1}) and streptomycin (100 µg ml^{-1}). Human adenocarcinoma colorectal cells (HT-29), were cultured using complete (DMEM) media. Cells were passaged every 36 h at 80% confluent. The cultured cells were incubated at 5% CO_2 and 37°C.

Cell viability assay

The cytotoxic action of different doses of free 5-FU/LV combination and 5-FU/LV-loaded NPs treatments was investigated using an MTT cell viability assay [58]. In 24-well plates, 5.0×10^4 cells were seeded per well for the MTT assay. The cells were then treated with different concentrations of free 5-FU/LV and 5-FU/LV-loaded NPs suspended in Optimem® media on the next day. Control cells with no treatment were served considered a negative control. Cells treated with blank Eudragit S100 NPs suspension were considered a positive control. The cytotoxic effect of different concentrations of free 5-FU/LV combination and 5-FU/LV-loaded NPs at concentrations of 30/10, 60/20, and 90/30 µM was detected every 12 h to estimate cancerous cells viability. The treated cancer cells were washed with PBS, then a 15% solution of MTT dye. The seeded plates were incubated at 37°C and 5% CO_2 for 3 h. The cell supernatants were removed, and formazan crystals were solubilized using DMSO. The absorptive wavelength was measured at λ_{max} of 570 nm in a microplate reader (Fluostar Omega, BMG Lab Tech GMBH, Munster, Germany). The untreated control cells absorption spectrum was set at 100%. Cell viability was calculated for each treatment as a percentage of cell growth compared to control cells. All of the experimental tests were carried out three times.

Statistical analysis

The resulting data for *in vitro* studies are described as mean ± standard deviation (SD). Graphpad prism® version 7.0 (GraphPad Software, CA, USA) was used for data analysis. A one-way analysis of variance (ANOVA) was performed to compare the mean values between the studied groups. $p \leq 0.05$ was considered as significant.

Results

Effect of PVA concentration of the external aqueous phase

PVA has a crucial role as a stabilizing agent for the secondary emulsion aqueous phase, showing the small and homogeneous distribution of the prepared polymeric nanoparticles [57]. The physicochemical characteristics of three different formulations of 5-FU/LV-loaded NPs (F1, F2 and F3) (Table 1), made by varying the external aqueous phase PVA concentration, are presented in Figure 1. Results showing the variable concentrations of PVA-affected drug-loaded NPs size are given in Figure 1A. The increase in PVA concentration from 0.5% w/v (F1) to 1% w/v (F3) resulted in a significant decrease in NP size ($p < 0.01$). Also increasing PVA concentration to 0.75% w/v showed a significant decrease in NP size ($p < 0.05$). In addition, increasing PVA concentration from 0.5% to 1% w/v in the external aqueous phase showed a significant decrease in PDI ($p < 0.05$) (Figure 1B). The lowest NP size 405.0 nm and PDI of 0.33 were reported for F3.

Effect of polymer amount in the organic phase

The change in polymer concentration exhibits an obvious effect on 5-FU/LV-loaded NPs physicochemical characterization (F3, F4 and F5), which is shown in Figure 2. Increasing the polymer amount in the same organic phase

Table 1. Different formulation variables for the preparation of 5-fluorouracil/leucovorin-loaded nanoparticles.

Formula	PVA % (w/v)	Polymer amount (mg)	Organic phase volume (ml)	Internal phase volume (ml)	External phase volume (ml)
F1	0.5	50	5	0.3	20
F2	0.75	50	5	0.3	20
F3	1	50	5	0.3	20
F4	1	75	5	0.3	20
F5	1	100	5	0.3	20
F6	1	50	3	0.3	20
F7	1	50	7	0.3	20
F8	1	50	5	0.3	30

PVA: Polyvinyl alcohol.

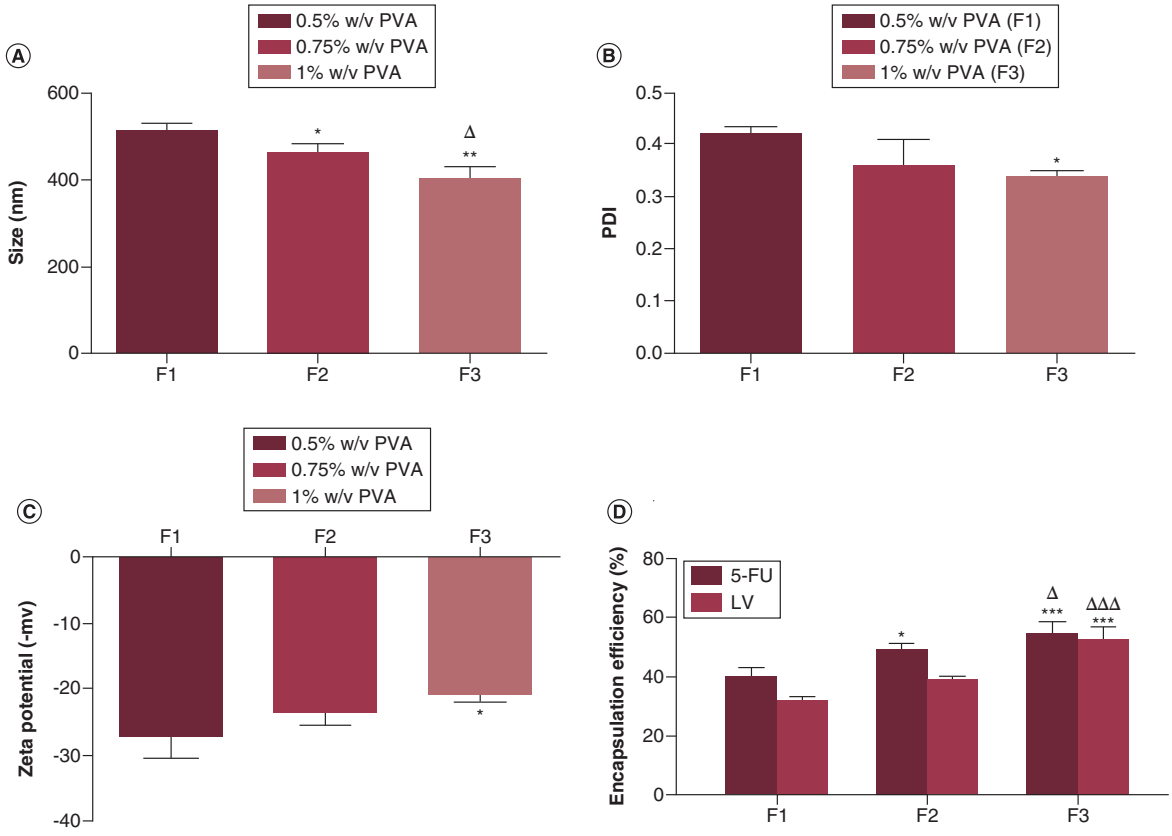

Figure 1. Effect of concentration on physical properties. The effect of PVA concentration in the external aqueous phase on 5-FU/LV-loaded NPs **(A)** size, **(B)** PDI, **(C)** zeta potential and **(D)** encapsulation efficiency of 5-FU and LV. Results are mean ± standard deviation and n = 3.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared with F1.
$^{\Delta}p < 0.05$; $^{\Delta\Delta}p < 0.01$; $^{\Delta\Delta\Delta}p < 0.001$ compared with F2.
FU: Fluorouracil; LV: Leucovorin; PDI: Polydispersity index; PVA: Polyvinyl alcohol.

volume shows a significant increase ($p < 0.01$) in the size of the loaded nanoparticles. The size of 5-FU/LV-loaded NPs increased significantly from 405.0 nm (F3) to 555.56 nm (F5) ($p < 0.01$) (Figure 2A) because of the elevation of the polymer content from 50 mg to 100 mg. Increasing the polymer amount showed a non-significant impact on the nanoparticles' size distribution and PDI ($p > 0.05$) (Figure 2B). Increasing polymer amounts substantially enhanced the surface charge from -20.6 mV (F3) to -26.67 mV (F5) ($p < 0.05$). It was found that the encapsulation efficiency (%EE) of both drugs significantly increased by increasing polymer amount in the organic phase

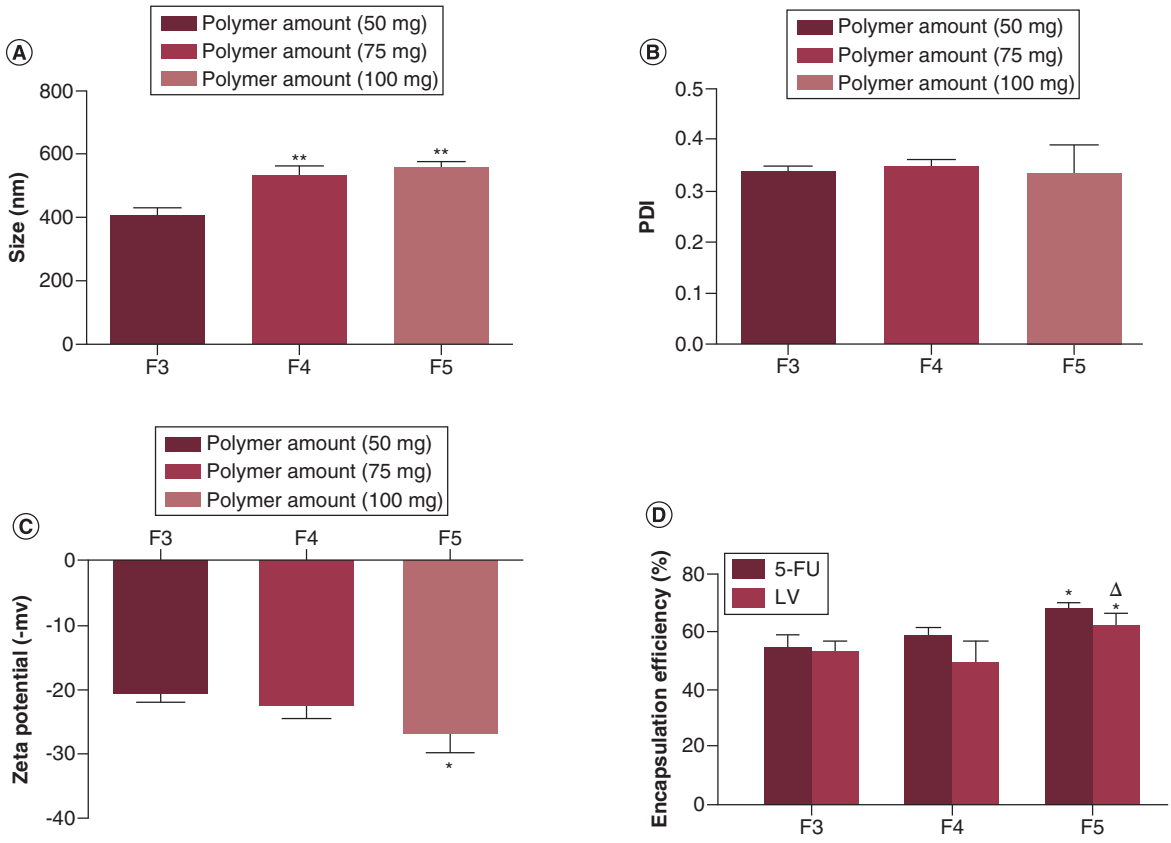

Figure 2. The effect of polymer amount on 5-fluorouracil/leucovorin-loaded nanoparticles. (A) Size, **(B)** PDI, **(C)** zeta potential and **(D)** encapsulation efficiency of 5-FU and LV. Results are mean ± standard deviation and n = 3.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared with F3.
$^{\Delta}p < 0.05$; $^{\Delta\Delta}p < 0.01$; $^{\Delta\Delta\Delta}p < 0.001$ compared with F4.
FU: Fluorouracil; LV: Leucovorin; PDI: Polydispersity index.

($p < 0.05$) (Figure 2D). The %EE of LV increased from 31.86% in F1 to 52.94% in F3 ($p < 0.001$). Also, %EE of 5-FU increased from 39.9% in F1 to 54.46% at F3 ($p < 0.001$) (Figure 1D).

Effect of O/W2 ratio

To ensure perfect solubility of the Eudragit S100 polymer, an organic solvent mixture of methanol and DCM at a ratio of 2:1 v/v was utilized. In this section, we used different O/W2 ratios to investigate their effects on the droplet sizes of W1/O/W2 emulsions and hence the final NP size. The physicochemical characterizations of F5, F6, F7 and F8 are represented in Figure 3. As shown in Figure 3A, there are significant differences in these formulas' nanoparticle sizes. Decreasing the O/W2 ratio by decreasing the organic solvent volume to 3 ml in F6 compared to 5 ml in F5 showed a sharp decrease in particle size from 555.56 nm (F5) to 160.40 nm (F6) ($p < 0.001$). Similar results were obtained by lowering the O/W2 ratio through another approach continuing to increase the external aqueous phase volume to 30 ml in the case of (F8) and keeping the organic phase volume constant. F8 showed a significantly lower size (264.55 nm) compared to both F5 and F7 ($p < 0.001$). On the other hand, increasing the O/W2 ratio by increasing the organic phase volume to 7 ml in F7 exhibited a larger NP size. Increasing the O/W2 ratio exhibited a significant increase in NP size $p < 0.05$ compared to F5 (Figure 3B). The used mixture, methanol (miscible with water) and DCM (immiscible with water), shows some aggregations that can not be completely stabilized with PVA and thus yield large-sized NPs [59].

The change of the O/W2 ratio showed a significant effect on zeta potential. A reported decrease in zeta potential for F6 ($p < 0.05$) and F8 ($p < 0.01$) after decreasing the O/W2 ratio was observed compared to F7 with higher O/W2 ratio (Figure 3C). The encapsulation efficiency of 5-FU and LV increased from 67.78% and 62.41% in the case of F5 to 85.45% and 74.04% in the case of F6 ($p < 0.01$).

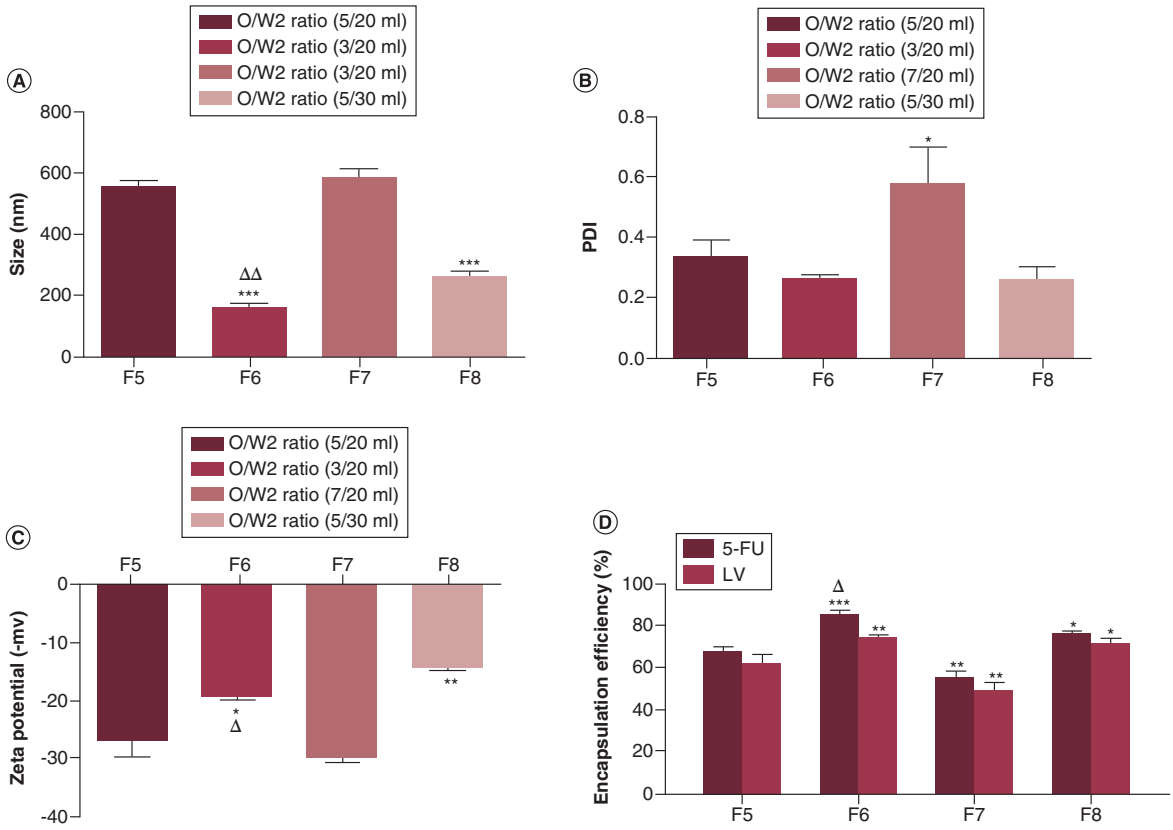

Figure 3. The effect of oil/water2 ratio on 5-fluorouracil/leucovorin-loaded nanoparticles. (A) Size, **(B)** PDI, **(C)** zeta potential and **(D)** encapsulation efficiency of 5-FU and LV. Results are mean ± standard deviation and n = 3.
*p < 0.05; **p < 0.01; ***p < 0.001 compared with F5.
$^{\Delta}$p < 0.05; $^{\Delta\Delta}$p < 0.01; $^{\Delta\Delta\Delta}$p < 0.001 compared with F8.
FU: Fluorouracil; LV: Leucovorin; O/W: Oil/water; PDI: Polydispersity index; PVA: Polyvinyl alcohol.

The previous results indicated that 5-FU/LV-loaded NPs (F6) and (F8) showed superior physicochemical properties to other NPs. Both F6 and F8 exhibited lower nanosize, lower PDI and higher encapsulation efficiency than other formulas. However, F6 showed significant smaller nanosize ($p < 0.01$) and a significantly higher surface charge ($p < 0.05$) than F8. Besides, F6 exhibits a marked increase in 5-FU encapsulation efficiency compared to F8 ($p < 0.05$).

Representative dynamic light scattering (DLS) graphs of F6 and F8 (Figure 4) showed good homogeneity and distribution of F6 compared to F8, which showed some aggregates of bigger size (>1 µm) (Figure 4B). These aggregates might be related to F8's significantly lower zeta potential than F6. This finding indicates the higher F6 stability. Therefore, F6 was selected as the optimum 5-FU/LV-loaded NPs due to its lower size, lower PDI, higher encapsulation efficiency of both drugs and moderate zeta potential compared with the other prepared NPs.

Surface morphology of 5-FU/LV NPs

Figure 5 illustrates the scanning electron microscopy (SEM) images of 5-FU/LV-loaded NPs (F6). The scanned NPs showed a smooth spherical shape of monodisperse NPs. There are no aggregates and the size is uniform. The mean size determined by SEM was comparable to that determined by the zeta sizer.

In vitro release of drug vs time

In simulated gastrointestinal fluids, the *in vitro* drug-release profiles of 5-FU and LV from the 5-FU/LV-loaded NPs (F6) were determined. It can be observed from Figure 6 that the drugs released from the Eudragit S100 NPs can be separated into four different stages. At the primary stage, very low 5-FU and LV amounts were released from the NPs in the simulated gastric fluid (0–2 h). After the first 2 h of *in vitro* release, approximately 15.2% and 10.1% of 5-FU and LV, respectively were released from drug-loaded NPs. At the subsequent secondary stage, when the

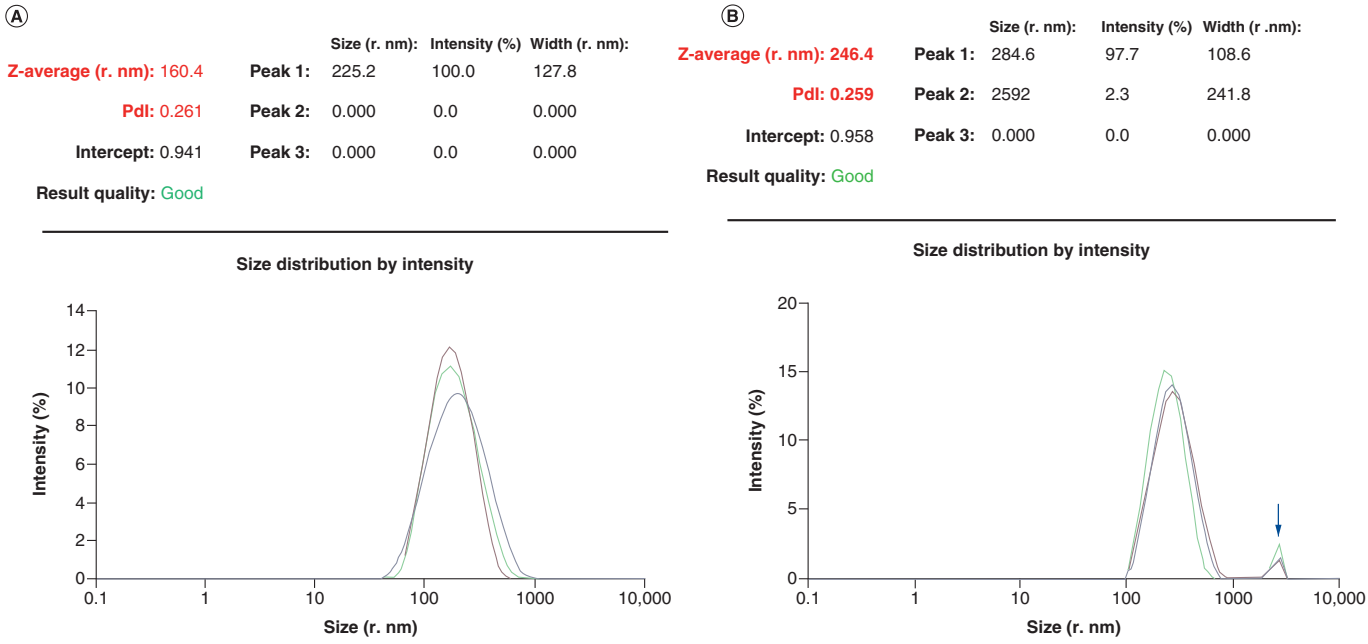

Figure 4. Illustrations of representative graphs of the distribution of particle size of 5-fluorouracil/leucovorin-loaded nanoparticles. (A) F6 and **(B)** F8. Aggregation was pointed through size analysis (blue arrow), which was absent in F6.
PDI: Polydispersity index.

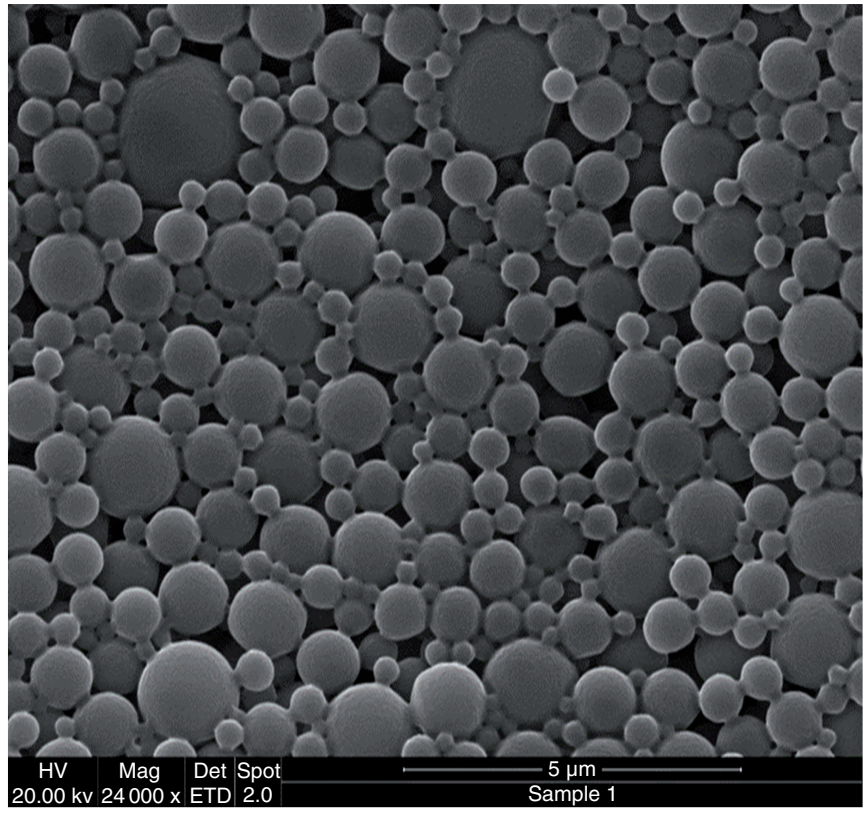

Figure 5. Scanning electron microscope image of optimized 5-fluorouracil/leucovorin-loaded nanoparticles (F6).

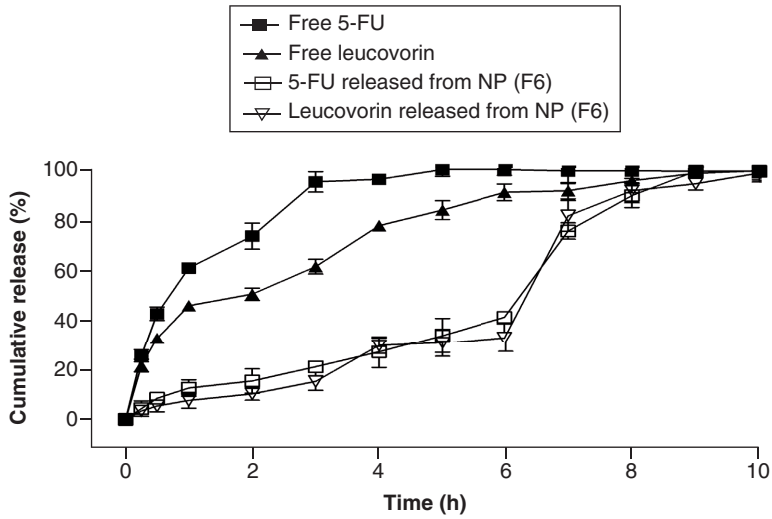

Figure 6. Cumulative release of 5-fluorouracil and leucovorin from 5-fluorouracil/leucovorin-loaded nanoparticles (F6) at different simulated gastrointestinal fluids.
FU: Fluorouracil; NP: Nanoparticle.

pH level reached 4.5, there was a slow drug liberation in the simulated gastric and intestinal fluids. Within 2–4 h, released 5-FU and LV started to increase steadily; 26.8 % and 29.4 % respectively were released after the first 4 h from 5-FU and LV. At the third stage, due to the enteric coating effusion properties of the Eudragit S100, both drugs were released slowly to reach 40.85 % and 32.58 % of 5-FU and LV, respectively after 6 h. In the final stage, a bursty release occurred with an overall releasing percentage of 89.34 % and 91.24 % of 5-FU and LV, respectively due to the complete dissolution of the Eudragit S100 enteric polymer which resulted in both drugs rapid release at a pH of 7.4. The drug-loaded NPs can control the release of both drugs constantly and slowly as the pH of the simulated gastric and intestinal fluids is less than 7 [44].

In vitro cytotoxicity

The cytotoxic action of different doses of free 5-FU/LV combination and 5-FU/LV-loaded NPs treatments on CT26 colonic cancer cells and HT-29 colorectal cancer cells were assessed by MTT assay at particular time intervals of 12, 24, and 36 h of NPs treatment (Figure 7) Blank Eudragit S100 NPs had almost no cytotoxic effect on both cancer cells. The dose–response graphs were plotted to expose the IC_{50}, which is the treatment concentration that results in a 50% inhibitory activity of cancer cell growth. Treatment with a free 5-FU/LV combination showed a significant reduction in cell viability of each of the cell lines compared to control ($p < 0.05$). In addition, the free 5-FU/LV combination seems to be affected by dose as cell viability decreased with increasing the dose from 30/10 to 90/30 μM.

Discussion

Emulsification/solvent evaporation method is one of the most reliable methods for preparing MPs or NPs [60]. The current research adopted it because it can be used for the nanoencapsulation of a wide range of active pharmaceutical drugs relying on their aqueous solubility. The emulsification of the aqueous drug and polymer organic phases followed by the organic solvent evaporation had resulted in polymeric phase solidification in the presence of the drug entrapped inside the prepared MPs/NPs [61]. This work is designed to study the effect of various formulation parameters on the physicochemical properties of the prepared 5-FU/LV-loaded NP. Fabrication process variables including the PVA concentrations in the external aqueous phase, polymer amount, and the O/W2 ratio, were used to optimize 5-FU/LV-loaded NP preparation. The influences of these variables on particle size, zeta potential, NP surface shape, encapsulation efficiency and the *in vitro* release profile were studied.

PVA can increase the degree of viscosity of the external aqueous phase, which will result in smaller NPs with large surface area [32]. The shear forces resulting from the sonication process during the formation of nanodroplets will prevent coalescence between nanoparticles [32]. In addition, increased viscosity improves the emulsion stabilization, inhibits the formation of premature aggregation of nanodroplets, and keeps the small and narrow size of the formed nanoparticles after solidification [62]. Increased PVA concentration exhibited a significant decrease in NPs surface charge from -27.15 mv in the case of F1 to -20.6 mv in F3 ($p < 0.05$) (Figure 1C). The PVA coating of the NPs surface with a thin-layer reduces the zeta potential. Elevation in PVA concentration leads to PVA depositing film

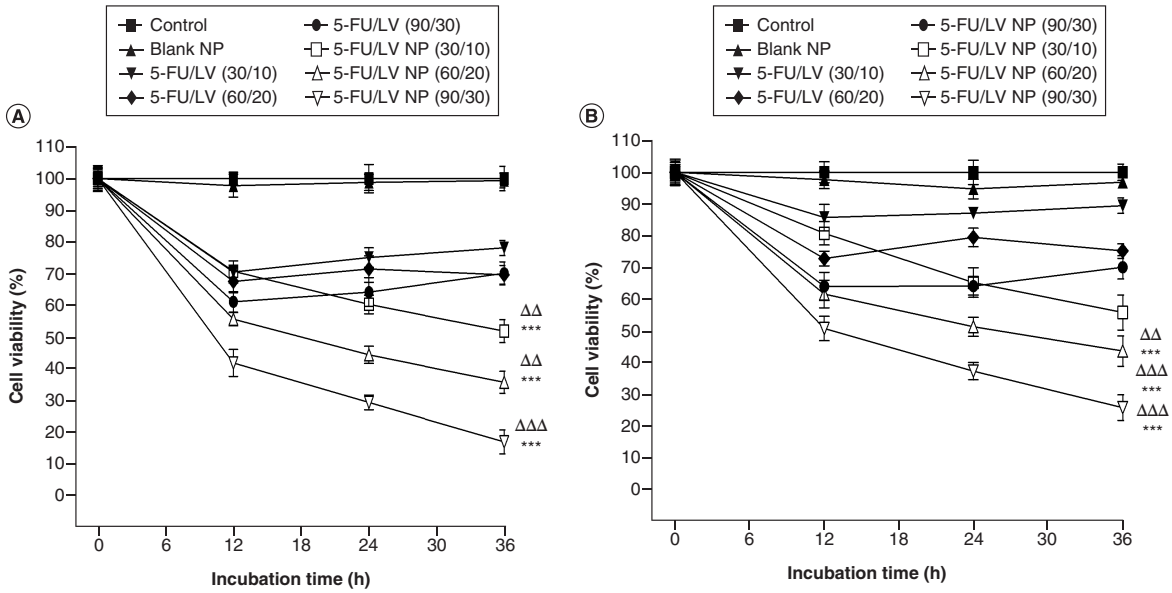

Figure 7. Cell viability assessment. Results of cell viability of different doses of free 5-FU & LV and 5-FU/LV-loaded NPs (F6) after 12 h, 24 h and 3 days of incubation with various treatments using CT-26 cell line **(A)** and HT-29 cell line **(B)** measured with MTT assay. Results are represented as mean ± standard deviation for (n = 3).
***$p < 0.001$ compared with control and blank NPs.
$^{\Delta\Delta}$ $p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$ in contrast to free 5-FU and LV at the same dose.
FU: Fluorouracil; LV: Leucovorin; NP: Nanoparticle; PDI: Polydispersity index.

which masks the polymer negative charge. Also, the encapsulation efficiency significantly increases upon increasing the external aqueous phase PVA concentration. The %EE of LV increased from 31.86% in F1 to 52.94% in F3 ($p < 0.001$). Also, %EE of 5-FU increased from 39.9% in F1 to 54.46% at F3 ($p < 0.001$) (Figure 1D). This finding could be attributed to the increased viscosity of secondary emulsion which impairs drugs diffusion from the inner phase to the water outer phase via the polymeric phase, facilitating hydrophilic drug entrapment within the polymer matrix [44,58,59].

Eudragit S100 is a methylmethacrylate anionic co-polymer with many advantages, including site targeting ability at the colon due to solubility at specific colonic pH, biodegradability, and biocompatibility. The increase in 5-FU/LV-loaded Eudragit S100 NPs could be explained by the high viscosity of organic phase because of high polymer concentration. The higher the viscosity of the polymer organic phase, the larger the size of the dispersed droplets formed in the secondary emulsion [63]. The negatively charged F5 zeta potential was significantly increased by the increase of the amount of Eudragit S100 polymer to 100 mg (Figure 2C). In addition, the enhancement in zeta potential may depend on the accumulation of ionized carboxylic groups on the NPs surface as the amount of Eudragit in the organic phase increases. It was demonstrated that the zeta potential value, which directly affects the stability of formed NP when it increases in value, may be explained by the repelling forces between NPs [18]. The enhancement in %EE may also be regarded to the higher viscosity degree of the polymeric organic phase that prevents diffusion of the internal aqueous phase containing both drugs. In addition, the highly concentrated polymer can be deposited rapidly after organic solvent removal leading to rapid polymer phase solidification that can prevent drug leakage to the outer aqueous phase [27].

The organic solvent type influences the physicochemical properties of NP, especially particle size [61]. The particle size is affected directly by the miscibility degree of organic solvent with water in double emulsion formation and how long it can stay at the liquid–liquid interface before evaporation. The sharp decrease in particle size from 555.56 nm (F5) to 160.40 nm (F6) could be due to the formation of relatively smaller emulsion droplets. which resulted in smaller drug-loaded NPs after solidification. Increasing the O/W2 ratio exhibited a significant increase in NP size could be explained by creating larger emulsion droplets and the immiscibility of DCM with water, which causes the particles with the internal aqueous phase to aggregate and form larger particles [59]. The decrease in zeta potential was observed after decreasing the O/W2 ratio. These findings are consistent with previous findings which were reported by other researchers [61,63]. As the ratio between the oily phase and external aqueous phase decreased,

the zeta potential (mV) decreased. The change in the O/W2 ratio shows a significant effect on 5-FU and LV encapsulation efficiency (Figure 3D). The increase in encapsulation efficiency can be due to an increase in organic solvent evaporation rate, which resulted in rapid polymeric phase solidification, facilitating active constituents entrapment within the nanoparticles [64].

The *in vitro* release results depicted that the nanoencapsulation of both drugs using Eudragit S100 colon targeting polymer can decrease the exposure of the drug-loaded NPs in the upper gastrointestinal tract. On the other hand, At the colon pH, the liberation of the drugs was observed to be rapid as a result of both dissolutions of the drug and complete digestion of the NP matrix [65].

Treatment with 5-FU/LV-loaded NPs (F6) decreases cell survival in a dose-dependent manner; meanwhile, it preserved its cytotoxic activity up to 36 h following treatment administration due to controlled drug release. The mean IC_{50} value for 5-FU/LV-loaded NPs (F6) in CT26 cells was approximately 30/10 μM and was achieved after 36 h. On the other hand, the IC_{50} for 5-FU/LV-loaded NPs (F6) in HT-29 cells was approximately 60/20 μM, which was accomplished after 24 h of treatment. The highest dose of treatment (90/30 μM) of drug-loaded NPs demonstrated a significant reduction in the cell viability of 83% and 74% in the case of CT-26 and HT-29, respectively, in comparison to free drug combination ($p < 0.001$). These results showed that 5-FU/LV-loaded NPs can deliver both drugs to their subcellular site for cancer cells to reach reduced cell viability and higher cytotoxic effect when compared with the free drug combination.

Conclusion

Codelivery of two or more drugs loaded into nano-drug delivery carriers for cancer treatment has been proposed to maximize drug-delivery effectiveness and safety to the target cancer site. The combined parenteral 5-FU and LV is the standard treatment for colon and colorectal cancers. In the current study, we optimized the preparation of 5-FU/LV-loaded Eudragit S100 NPs for the oral delivery of both drugs to the CC and CRC. Eudragit S100 has a promising role in drug targeting to the colon via its pH-responsive properties. Eudragit S-100 NPs loaded with 5-FU/LV drugs were fabricated with a modified double emulsion solvent evaporation technique through some formulation variables such as PVA concentration, polymer amounts and the ratio of organic solvent to external phase volume. The higher PVA concentration in the outer aqueous phase has significantly played a role in stabilizing the prepared formula by decreasing the nanoparticles' size, PDI and increasing the drug entrapment. Deceasing the O/W2 ratio decreases the occurrence of coalescence that affects the secondary emulsion formation and hence decreases the size while enhancing the encapsulation efficiency of both drugs. An optimum formula, F6, was selected based on its smaller particle size, 160.4 nm, higher %EE of both drugs, lower PDI (0.265) and stable higher zeta potential than other formulas.

This study shows for the first time the preparation and optimization of 5-FU/LV-loaded nanoparticles, which efficiently improved its interaction with colon and colorectal cancer cells *in vitro*. The potential clinical interest of this study will be clearly achievable after *in vivo* studies. According to the *in vitro* results, the prepared colon targeted nanosystem can protect the drug release through gastrointestinal regions and enhances its release at colorectal sites, which enhances the anti-cancer activity of the free drug combination to a high extent. This strategy could ultimately reduce the dose of the anti-cancer drugs while providing improved therapeutic outcomes, which will minimize the side effects of the drug on the patient. The authors are planning to perform *in vivo* animal studies to reflect the clinical importance of colon targeting of 5-FU/LV chemotherapeutic combination through Eudragit S100 nanoparticles.

Future perspective

The *in vitro* release showed that optimized drug-loaded NPs could act as a carrier to minimize drug release against gastric and intestinal pH environments, thus protecting the drugs against the pathway of digestion to reach the preferred site of action at the colon and rectum. Drug-loaded NPs showed a significant cytotoxic action on colon and colorectal cancer cell lines *in vitro* compared to free drug combinations. These novel drug-loaded NPs can be further tested in pre-clinical models and clinical trials to develop an effective and promising therapeutic tool for the oral therapy of colon and colorectal cancers.

Summary points

- The chemotherapeutic combination of 5-fluorouracil (FU) and leucovorin (LV) loaded with Eudragit® S100 nanoparticles (NPs) was tested for colon cancer targeting.
- A modified double emulsion solvent evaporation technique was used for the preparation of the 5-FU/LV-loaded Eudragit S100 NPs suitable for oral delivery.
- Optimization of the prepared 5-FU/LV-loaded Eudragit S100 NPs was achieved through different formulation variables such as polyvinyl alcohol (PVA) concentration, amount of polymer and organic solvent to external phase volume ratio (O/W2).
- The fabricated 5-FU/LV-loaded NPs were smooth, spherical, monodisperse and negatively charged with a particle size range of 160–514 nm.
- The optimized formula (F6) was selected based on its smallest particle size of 160.4 nm, lowest PDI of 0.265, higher zeta potential and encapsulation efficiency of both drugs.
- The *in vitro* cytotoxic action of the optimum drug-loaded NPs was investigated using both colon cancer (CC) and colorectal cancer (CRC) cell lines.
- The release study showed that optimized drug-loaded NPs can act as a carrier to minimize *in vitro* drug release against simulated gastric and intestinal pH, meanwhile, maximize drug release at colonic pH.
- Drug-loaded NPs showed a significant cytotoxic action on colon and colorectal cancer cell lines *in vitro* compared to free drug combinations.
- These designed drug-loaded NPs can be used as an effective and promising therapeutic tool for the oral therapy of colon and colorectal cancers.

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