



Weak complexation of 5-fluorouracil with β -cyclodextrin, carbonate, and dianhydride crosslinked β -cyclodextrin: *in vitro* and *in silico* studies

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Abstract

Background and purpose: Several pharmaceutical formulations were investigated to improve the solubility of 5-fluorouracil to enhance bioavailability and therapeutic efficacy. This study aimed to examine the potential use of cyclodextrin-based nanosponges for the incorporation of 5-fluorouracil and to investigate the use of different crosslinking agents on the properties of the resulting drug carrier. 5-Fluorouracil complexation with β -cyclodextrin was also studied to explain the unexpected results of weak 5-fluorouracil incorporation in nanosponge.

Experimental approach: Nanosponges were synthesized by crosslinking β -cyclodextrin with two different crosslinkers; diphenyl carbonate and ethylenediaminetetraacetic dianhydride. The incorporation of 5-fluorouracil into β -cyclodextrin and the prepared nanosponges were assessed by NMR, FTIR, PXRD, DSC, and TGA. In addition, an *in vitro* release study was carried out to evaluate the potential use of β -cyclodextrin-based nanosponges as pharmaceutical formulations for 5-fluorouracil.

Findings / Results: Physicochemical characterization of the dried formulations indicated the complexation of 5-fluorouracil with the β -cyclodextrin polymer. Despite that, no clear manifestation of 5-fluorouracil encapsulation in the prepared β -cyclodextrin-based nanosponge was detected. Furthermore, no significant differences were observed in the release profiles of 5-fluorouracil, β -cyclodextrin complex, and β -cyclodextrin-based nanosponge, suggesting weak complexation and instability in aqueous solutions. EDTA-crosslinked β -cyclodextrin-based nanosponge showed a slight improvement in 5-fluorouracil solubility with a faster initial rate of 5-fluorouracil release.

Conclusion and implications: This study suggested weak complexation between 5-fluorouracil and the β -cyclodextrin polymer or nanosponges. Crosslinking of β -cyclodextrin with EDTA dianhydride crosslinker showed an enhancement in 5-fluorouracil saturation solubility combined with a faster initial rate of drug release.

Keywords: β -Cyclodextrin-based nanosponges; Complexation; Crosslinking agent; 5-Fluorouracil.

INTRODUCTION

5-Fluorouracil (5-FU) is a nucleobase analog derived from uracil where a fluorine atom replaces the hydrogen at position 5 (Fig. 1A) (1). 5-FU is a potent cytotoxic drug that blocks DNA synthesis by inhibiting thymidylate synthase, the enzyme responsible for converting deoxyuridylic acid to thymidylic

acid (1,2). 5-FU is one of the first-line chemotherapeutic agents for the treatment of metastatic colorectal cancer (3).

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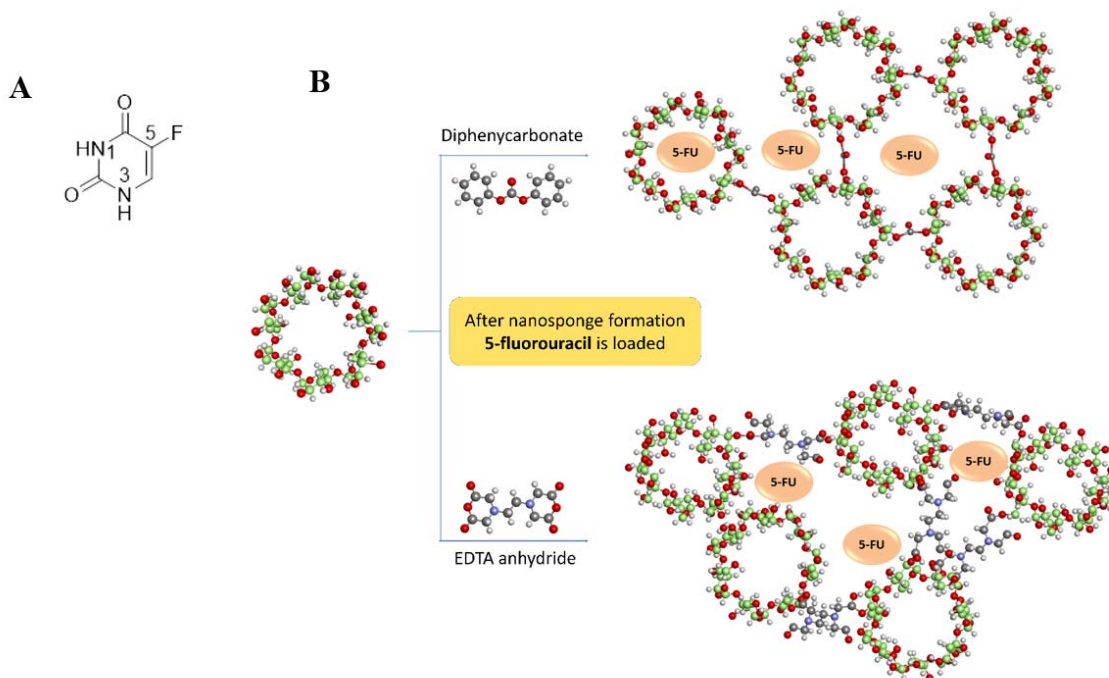


Fig. 1. (A) Chemical structure of 5-fluorouracil and (B) schematic representations of the 5-FU-loaded diphenyl carbonate and EDTA dianhydride crosslinked nanosponges. EDTA, Ethylenediaminetetraacetic acid, FU, fluorouracil.

Oral administration of 5-FU was restricted due to its relatively low aqueous solubility, which was reported to range between 7.4 and 12.5 mg/mL in water and 17.6 mg/mL in phosphate buffer (4). Furthermore, erratic intestinal absorption and variation in its bioavailability were observed (3).

Nanosponge is referred to as a highly porous carrier with sponge-like structures. Generally, cyclodextrin nanosponges are insoluble carriers of drugs whose covalent networks are formed by intertwining cyclodextrin polymers in three dimensions and creating nanochannels with nanopores. By being stable, insoluble, biocompatible, and able to encapsulate active pharmaceutical agents through the formation of inclusion and non-inclusion complexes, nanosponges have attracted attention for drug delivery applications (5). They are reported to enhance drug solubility, increase dermal permeability and retention (6), control the drug release rate, improve drug stability, and minimize degradation (7).

Cyclodextrins can be crosslinked using a wide range of multifunctional agents such as dianhydrides, carbonyl compounds, diisocyanates, carboxylic acids, and epoxides.

Crosslinker nature and degree largely influence the characteristics of the final nanosponges (8). Cyclodextrins, for example, can host hydrophobic ingredients within their inner cavities. Meanwhile, the formed interstitial pores between the crosslinker and the external edges of cyclodextrins explain how cyclodextrin nanosponge can load hydrophilic ingredients (5). Dianhydride crosslinked nanosponges were expected to incorporate drugs by two mechanisms; first, by the formation of inclusion complexes with inner cyclodextrin cavities, and second, in the hydrophilic cavities resulting between cyclodextrin units by the formation of electrostatic interactions with the free carboxylic functional groups resulting from dianhydride crosslinking of cyclodextrin (9).

The main objective of this study was to evaluate the use of ethylenediaminetetraacetic acid (EDTA) dianhydride cyclodextrin-based nanosponges which were expected to have more hydrophilic interstitial pores for the incorporation of 5-FU and to compare it to the mostly used diphenyl carbonate (DPC) crosslinked nanosponges.

MATERIALS AND METHODS

Materials

5-FU was provided by Hubei Vanz Pharm Co., Ltd. (China). β -Cyclodextrin polymer, anhydrous dimethyl sulfoxide (DMSO), triethylamine (TEA), and EDTA dianhydride were purchased from Sigma-Aldrich Chemical (USA), high-performance liquid chromatography (HPLC) grade water and absolute ethanol were provided from Honeywell (France). DPC was purchased from TCI Chemicals (Japan). Acetonitrile HPLC grade was provided by Scharlau (Spain). Acetone HPLC grade was provided by Lab Chem, (USA). Potassium phosphate dibasic was purchased from Xilong Chemical Industry (China). Potassium dihydrogen phosphate was provided by AZ Chem for chemicals (Canada). Deuterated DMSO- d_6 was purchased from Santa Cruz Biotechnology (USA).

The β -cyclodextrin powder was dehydrated until a constant weight was attained, utilizing a vacuum oven at 40 °C. All other materials were used as provided without any further modifications.

Preparation of β -cyclodextrin-crosslinked nanosponge

DPC-crosslinked nanosponge

β -cyclodextrin nanosponges were prepared by the solvent-free fusion method (10). In brief, 1 mM of β -cyclodextrin was added to a remelted 4 or 8 mM of DPC crosslinker for nanosponge (NS4) and nanosponge (NS8) preparations, respectively. The reaction was carried out in a paraffin oil bath at 90 °C, with continuous stirring at 200 rpm for 5 h. The resultant mass was crushed, washed with pure water, and then rewashed with acetone to remove any phenolic byproduct. The dried mass was allowed to air dry at room temperature.

EDTA-crosslinked nanosponge

Nanosponges were synthesized using a previously reported polymer condensation method with modifications using a 1:8 molar ratio of β -cyclodextrin to EDTA dianhydride crosslinker. In brief, 8 mM EDTA dianhydride crosslinker was added to a solution of 1 mM β -cyclodextrin powder in 5 mL anhydrous

DMSO (5 mL) and triethylamine (1 mL), and the mixture was stirred at 35 °C, with continuous stirring at 300 rpm. The resultant mass was crushed and washed with water (45 mL \times 2), and acetone (45 mL \times 3), then allowed to dry at room temperature (11).

Preparation of 5-FU β -cyclodextrin complex and 5-FU-loaded nanosponge

β -Cyclodextrin complex

An excess of 5-FU was added to an aqueous solution of 10 mM β -cyclodextrin polymer and the solution was shaken at 100 rpm for 24 h. Afterward, the dispersions were centrifuged for 10 min at 3500 rpm at 25 °C in a thermostatic water bath, and aliquots from the supernatant were diluted to measure the effect on 5-FU solubility. The remaining supernatant was further dried using a freeze dryer.

5-FU-loaded nanosponge

Nanosponge (200 mg) was dissolved in 20 mL deionized water, sonicated for 20 min, and then the excess 5-FU was added and shaken at 100 rpm for 24 h. The dispersions were centrifuged at 3500 rpm for 10 min at 25 °C in a thermostatic water bath, and aliquots from the resulting supernatants were diluted to measure the effect on 5-FU solubility. The remaining supernatants were dried with a freeze dryer. A schematic representation of cyclodextrin-based nanosponges loaded with 5-FU is shown in Fig. 1B.

Computational methods

5-FU was sketched using ChemBioDraw Ultra 12.0 and all other *in silico* steps were performed using Discovery Studio (DS) 2017 from BIOVIA Software Inc. (12).

Preparation of 5-FU and β -cyclodextrin structures

The chemical structure of 5-FU was drawn and geometrically optimized using ChemDraw 12. Then, it was imported to Discovery Studio to generate different ionization states and to calculate 3D coordinates using the Prepare Ligands protocol. For β -cyclodextrin, the 3D coordinates were obtained from the crystal structure of a mutant alpha-hemolysin bound to β -cyclodextrin (PDB entry code 3M4E with a resolution of 2.3 Å) (13).

Preparation of 5-FU- β -cyclodextrin complexes

The 5-FU- β -cyclodextrin complexes were obtained by docking the prepared 5-FU into β -cyclodextrin. First, the β -cyclodextrin molecule was defined as a receptor where a site sphere of 10 Å radius surrounded the entire molecule, then the CDocker docking protocol (14) within Discovery Studio was run using default parameters. Finally, for running molecular dynamics (MD) simulations, the top-ranked docked poses for the neutral and ionized 5-FU in complex with β -cyclodextrin were selected and typed using the simulation tools by applying the CHARMM force field, and they were ready for running MD simulations.

MD simulations

In order to investigate the complexation stability of neutral and ionized 5-FU with β -cyclodextrin, MD simulations were utilized as detailed in the literature (15). Briefly, the two complexes (neutral 5-FU- β -cyclodextrin and ionized 5-FU- β -cyclodextrin) were solvated by immersing them in a truncated octahedral box of pre-equilibrated TIP3P water (16). Then, the solvated systems were initially minimized using 1000 steps of steepest descent minimization followed by another 4000 steps of conjugate gradient minimization. Then, the minimized structure was gradually heated to 298 °K throughout 100 ps. Next, the minimized systems were equilibrated for another 100 ps to equilibrate the system at the target temperature. Finally, the MD production phase commenced, where the simulation was continued for 10 ns under isothermal-isobaric thermodynamic ensemble (NPT) conditions that best mimic the experimental environment under which our study was conducted. The long-range electrostatic interactions were treated by the particle mesh Ewald method (17) using a non-bonded cut-off value of 14 Å (18).

Physicochemical characterization:**Nuclear magnetic resonance spectroscopy:**

Proton nuclear magnetic resonance (¹H NMR) data were collected using a Bruker Avance Ultrashield 400 MHz NMR spectrometer (Bruker BioSpin, Switzerland). DMSO-*d*₆ was used as a solvent, and the resultant data were analyzed using

MestReNova version 12.0. Residual solvent signals were utilized for reference.

Zeta-potential measurements:

The sample's zeta potential in aqueous suspensions was analyzed using the Malvern zeta sizer (ZEN 3600) instrument at 25 °C, with a 173° detection angle to increase the sensitivity of Dynamic light scattering (19), each measurement was run in triplicate.

Fourier transform-infrared spectroscopy:

Fourier transform-infrared (FTIR) spectrums were recorded for 5-FU, 5-FU- β -cyclodextrin complex, 5-FU-loaded nanosponge, and the corresponding physical mixtures, utilizing an FTIR spectroscopy model, IR affinity-1, Shimadzu, Japan, in a wavenumber range of 400-4000 cm⁻¹. Preceding measurements samples were finely ground and blended with KBr using a pestle and mortar.

Powder diffraction pattern

Powder X-ray diffractometer (PXRD) Ultima IV diffractometer (Rigaku, Japan) was used to record the X-ray diffraction patterns for the raw materials, prepared formulations, and the corresponding physical mixtures of raw materials.

Differential scanning calorimetry

Differential scanning calorimetry (DSC) thermograms of 5-FU, 5-FU- β -cyclodextrin complex and 5-FU-loaded nanosponge were conducted using DSC 204 F1 Phonex, differential scanning calorimeter (Netzsch, Germany). Prepared formulations were placed into aluminum pans, under a continuous nitrogen flow, the temperature program was carried out between 30 °C to 400 °C, with a rate of 10 °C/min.

Thermogravimetric analysis

Thermogravimetric analysis (TGA) thermograms of 5-FU, 5-FU- β -cyclodextrin complex, 5-FU-loaded nanosponge, and the corresponding physical mixture were recorded utilizing Shimadzu TGA-50 under nitrogen at a temperature range of 30-400 °C and a heating rate of 10 °C /min.

Scanning electron microscopy

The morphological properties of raw 5-FU, 5-FU- β -cyclodextrin complex, 5-FU-loaded NS4, and 5-FU-loaded EDTA NS8 nanosponge were observed utilizing a Jeol-JSM-5300 scanning electron microscope (SEM, Tokyo, Japan).

Drug content

An accurately measured amount of 5-FU- β -cyclodextrin complex and 5-FU-loaded nanosponges was dissolved in acetonitrile and then sonicated to ensure complete extraction of the drugs, filtered, and analyzed using the HPLC method.

In vitro release experiments

An *in vitro* release study was carried out for the prepared drug-containing nanosponge, and drug solutions. The dialysis membrane diffusion method was used. Accurately weighed samples equivalent to 5 mg 5-FU were suspended in 3 mL of the dissolution media placed in 7 cm long dialysis membrane tubes (Visking, molecular weight cut-off 12-14000 Da, Medicell Membrane Ltd, UK), closed from both sides, inserted in 100 mL stimulatory release medium of phosphate buffer 0.01 M pH of 7.4 inside a sealed glass bottle, in a shaking water bath at a constant temperature of 37 °C, and rotating at 100 rpm. All experiments were repeated in triplicate.

Statistical analysis

All results are presented as mean \pm SD. To judge whether the alterations of 5-FU solubility upon complexation are more than expected by chance, a one-way ANOVA statistical examination followed by Tukey's multiple comparisons test was conducted by the use of GraphPad Prism 9.0.0 software. *P*-values \leq 0.05 were considered significant.

RESULTS

FTIR spectroscopy

5-FU exhibited characteristic peaks in the FTIR spectrum (Fig. 2A) at 1247 cm^{-1} for the C-F bond stretching vibration, at 1454 cm^{-1} for the C=C bond, at 1645 cm^{-1} for the carbonyl stretching (C=O), at 1722 cm^{-1} for the CO-NH

stretching vibration, and at 3130 cm^{-1} for NH stretching vibration. Similar bands have been reported in the literature for 5-FU (20).

The FTIR spectrum of β -cyclodextrins (Fig. 2B) displayed the characteristic peaks of cyclodextrin polymer. A broad peak between 3000 and 3500 cm^{-1} corresponds to the O-H group stretching, at 2933.7 cm^{-1} for the CH₂ group stretching vibration, an intense peak at 1155 cm^{-1} indicated a C-H stretching, at 1033 cm^{-1} due to the C-O-C stretching vibration, an intense peak at 947 cm^{-1} attributed to α -1,4-glycosidic bond vibration, and a characteristic peak at 858 cm^{-1} corresponding to pyridine glycosidic bond. The FTIR spectrum of β -cyclodextrins agrees with the reported spectra in the literature (21).

The FTIR spectrum of β -cyclodextrins complexed with 5-FU in comparison with the corresponding physical mixture represented as shown in Fig. 2C. The physical mixture spectrum exhibited the main characteristic peaks without significant deviations. However, the spectrum of β -cyclodextrins complexed with 5-FU showed a shift in the β -cyclodextrin bands toward lower or higher wavenumbers, namely 2937-2943, 1155-1161, and 1033-1028 cm^{-1} . These shifts, besides the intensity decrement of 5-FU intense peaks (especially peak at 1247 cm^{-1} corresponding to C-F bond stretching vibration).

The FTIR spectrum of nanosponge NS4 is shown in Fig. 2D. Plain nanosponge spectrum indicated the crosslinking of β -cyclodextrins polymer, as it presents the main characteristic bands that define β -cyclodextrin, besides the appearance of a new intense peak around 1751 cm^{-1} corresponding to the carbonyl group stretching vibration, and the observed increase in peak intensity at 1232 cm^{-1} that indicates the formation of additional C-O bonds. Similar observations were reported in the literature (22).

5-FU-loaded NS4 showed shifts in the wavenumbers of the characteristic peaks of the 5-FU (1722-1728, 1645-1666, and 1246-1249 cm^{-1}), and β -cyclodextrins polymer (2933-2935, and 1159-1153 cm^{-1}). These shifts can be attributed to the interaction between 5-FU and the cyclodextrin nanosponge.

The FTIR spectrum of 5-FU-loaded NS8 is displayed in Fig. 2E. It showed intense peaks characteristic of the β -cyclodextrin polymer, which were identical to the corresponding physical mixture without significant deviations in wavenumbers. The same observation can be noticed for 5-FU characteristic peaks.

FTIR spectrum of 5-FU-loaded NS8 crosslinked using EDTA dianhydride crosslinker (EDTA-NS8) is displayed in Fig. 2F. The spectrum of the corresponding

physical mixture indicated the crosslinking of β -cyclodextrins polymer, as the spectrum showed the main characteristic bands of β -cyclodextrin, besides the appearance of a new intense peak around 1734 cm^{-1} corresponding to carbonyl group stretching vibration. Compared to the physical mixture, 5-FU-loaded EDTA NS8 showed some deviations in cyclodextrin and nanosponge characteristic peaks toward lower and higher wavenumbers and indicated the interaction and the encapsulation of 5-FU with EDTA NS8.

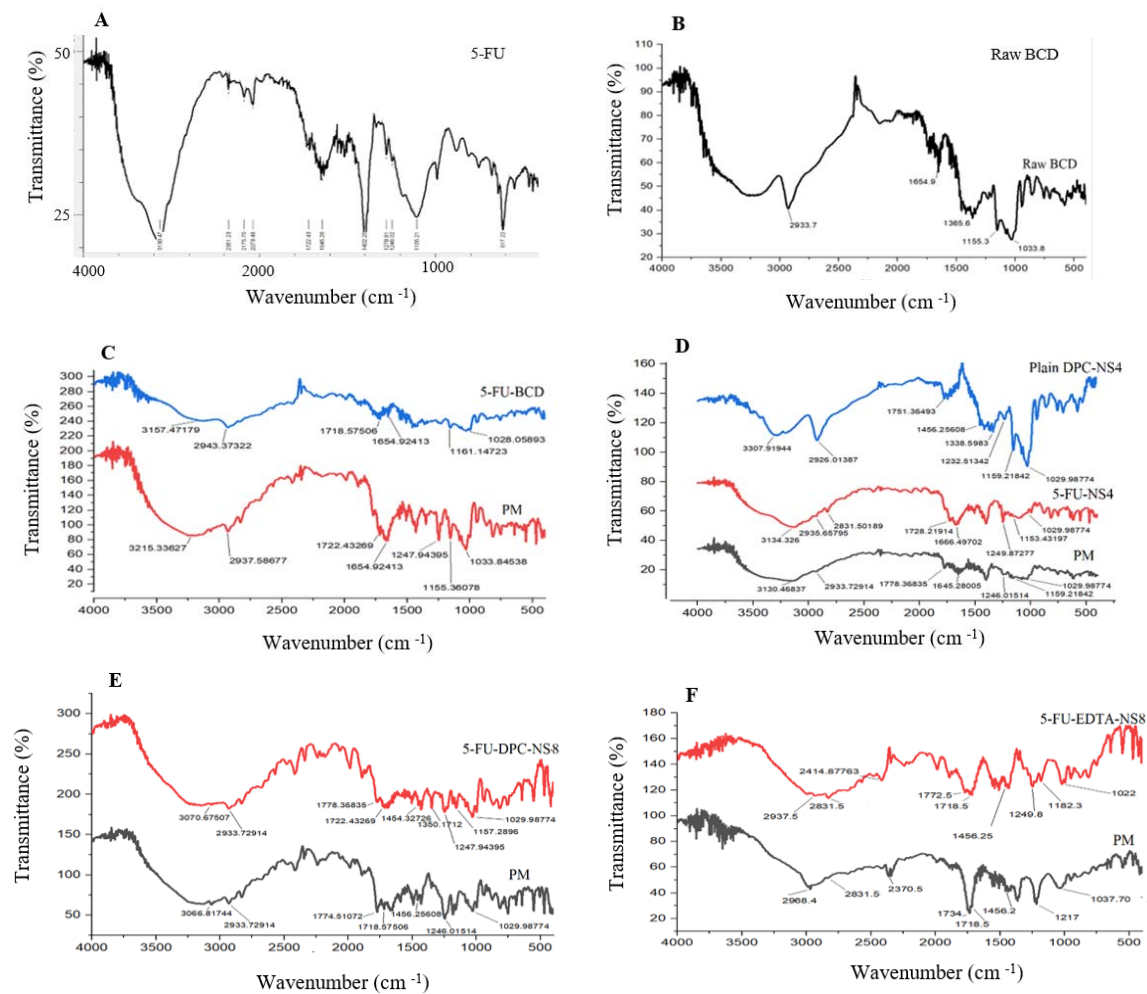


Fig. 2. Fourier transform-infrared spectrum of (A) raw 5-FU, (B) raw β -cyclodextrin, (C) 5-FU-loaded 10 mM BCD, (D) 5-FU-loaded DPC-NS4, (E) 5-FU-loaded DPC-NS8, and (F) 5-FU-loaded EDTA-NS8. BCD, β -cyclodextrin; FU, fluorouracil; PM, physical mixture; DPC, diphenyl carbonate; EDTA, ethylenediaminetetraacetic acid; NS, nanosponge.

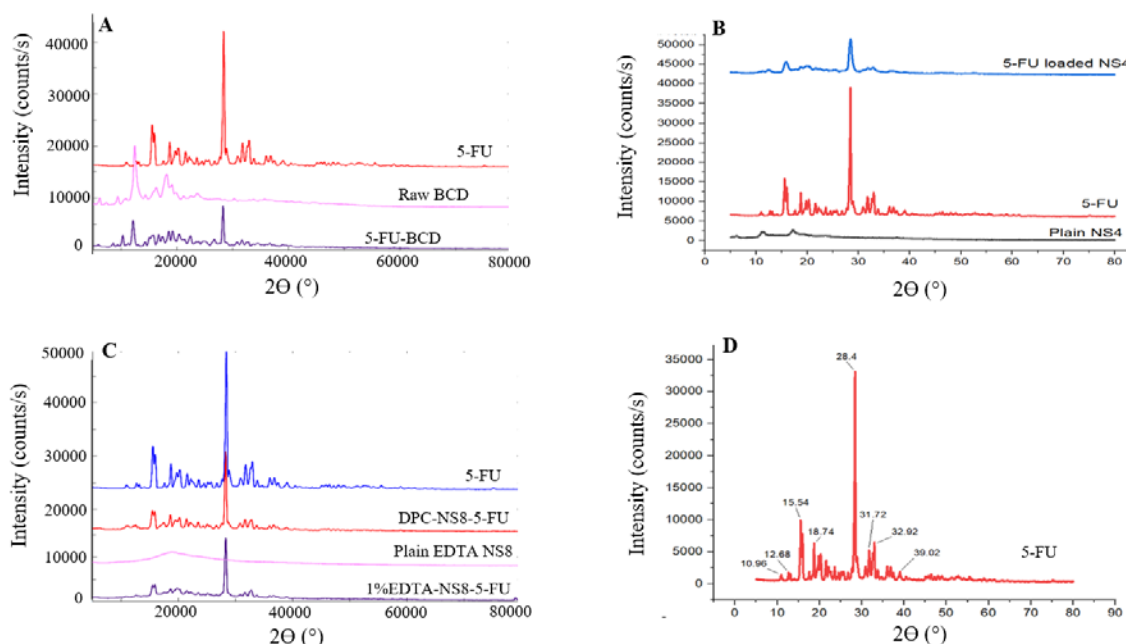


Fig. 3. Powder X-ray diffraction pattern of (A) 5-FU-loaded 10 mM BCD, (B) 5-FU-loaded DPC-NS4, (C) 5-FU-loaded DPC-NS8, and 5-FU loaded EDTA-NS8, and (D) raw 5-FU. BCD, β -cyclodextrin; FU, fluorouracil; DPC, diphenyl carbonate; EDTA, ethylenediaminetetraacetic acid; NS, nanosponge.

PXRD

The PXRD pattern of the 5-FU raw drug is shown in Fig. 3. The intense characteristic 5-FU peaks observed at 15.5, 18.7, and 28.4 degrees correspond to the crystalline polymorphic form 1 of 5-FU (23).

The PXRD pattern of cyclodextrin complexed with 5-FU (Fig. 3A) suggested the complexation as the characteristic peaks of 5-FU decreased, and cyclodextrin peaks mainly appear in the formed complex.

The PXRD patterns of 5-FU-loaded nanosponges NS4, NS-8, and EDTA nanosponge are displayed in Fig. 3B and C. The resultant patterns indicated a decrease in 5-FU crystallinity when loaded in nanosponge NS-4. However, the PXRD diffractogram of NS-8 confirmed the existence of 5-FU in the crystalline form in these formulations.

DSC

DSC thermogram of cyclodextrin complex is presented in Fig. 4. The sharp endothermic peak at 287.6 °C represents the melting point of 5-FU. This endothermic peak appeared at the same physical mixture position, suggesting the lack of physicochemical interaction between 5-FU and cyclodextrin. On the other hand, the

DSC thermogram of the formed complex showed a deviation in the 5-FU endothermic peak toward lower temperature, as this sharp peak appeared at 275 °C, confirming the formation of the inclusion complex.

The complete inclusion of crystalline drugs inside the cyclodextrin cavity is usually evident by the disappearance of the drug melting peak, as the formed complex is expected to display an amorphous DSC thermogram if all drug molecules are incorporated in the cyclodextrin cavity (24), and the appearance of a 5-FU endothermic melting peak in the formed complex suggested an incomplete inclusion of 5-FU inside the cyclodextrin cavity. The remarkable shift in the 5-FU endothermic peak in comparison to the raw 5-FU toward lower temperature (287.6-275.2 °C) is indicative of the presence of intermolecular forces between the drug and the cyclodextrin suggestive of complexation. Similar results were reported in previous studies for cyclodextrin complexation with other compounds (25).

The DSC thermograms of 5-FU-loaded nanosponges NS4, NS8, and EDTA nanosponges represented in Fig. 4B-D, drug-loaded nanosponge NS4 and the corresponding physical mixture exhibited broadening and

deviation in the endothermic peak toward lower temperature; 275.6 °C and 277.2 °C, respectively. Nanosponge NS4 and the corresponding physical mixture thermograms displayed an exothermic peak when the temperature just elevated higher than the 5-FU endothermic fusion peak, and this may indicate the exothermic energy of complexation interaction that occurs upon 5-FU melting, a similar exothermic peak was reported for the formed naproxen inclusion complex with β -cyclodextrin (24). The observed broadening of the endothermic peak attained in nanosponges thermograms can be related to the fusion between β -cyclodextrin-based nanosponge and 5-FU as the fusion of both materials overlying in adjacent positions as seen in Fig. 4E and F. A similar observation has

been outlined previously (26). Similar observations for broadening were reported for the physical mixtures of methyl β -cyclodextrin and 2-hydroxypropyl- β -cyclodextrin with an anti-HIV therapeutic agent (UC781) (27).

In agreement with XRD results, the DSC thermogram of plain EDTA-NS8 (Fig. 4A-D) confirms its amorphous nature, the DSC thermogram of drug-loaded EDTA NS8 exhibited a changed peak pattern compared to the raw drug, and the plain nanosponge, as the endothermic melting peak disappeared at 287.6 °C and new endothermic peaks came into sight at 284.2, 294, and 301.8 °C. Similar observation reported by Geng *et al.* study designated the strong interactions and the complex formation between bensulfuron-methyl and β -cyclodextrin (25).

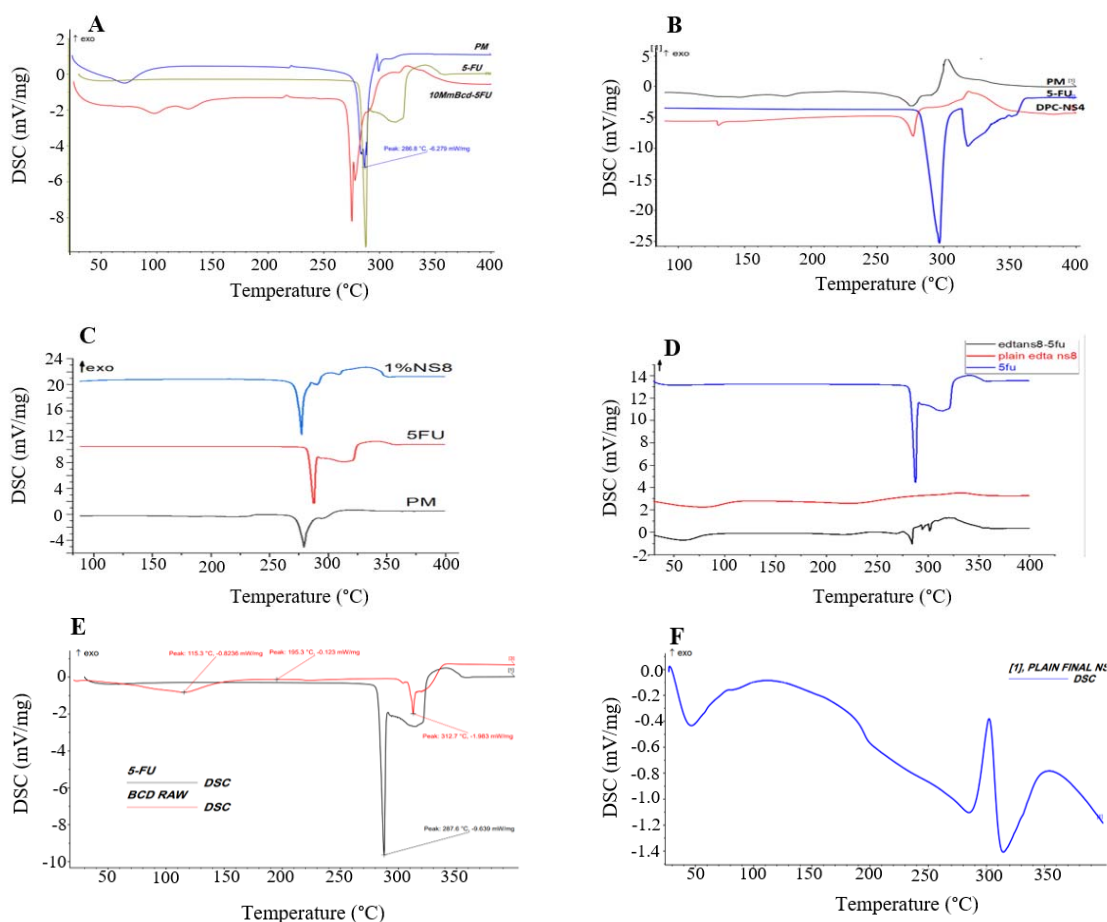


Fig. 4. DSC thermograms of (A) 5-FU- β -cyclodextrin complex, (B) 5-FU-loaded DPC-NS4, (C) 5-FU-loaded DPC-NS8, (D) 5-FU-loaded EDTA-NS8, (E) raw β -cyclodextrin and raw 5-FU, and (F) plain NS4 nanosponge. DSC, Differential scanning calorimetry; BCD, β -cyclodextrin; FU, fluorouracil; EDTA, ethylenediaminetetraacetic acid; NS, nanosponge; PM, physical mixture.

TGA

TGA curves of 5-FU-β-cyclodextrin complex, 5-FU-loaded nanosponge related to 5-FU and the corresponding physical mixture presented in Fig. 5, 5-FU curve displayed its stability up to 290 °C, the physical blend of 5-FU and plain nanosponge kept most of its mass (90%) up to 260 °C. Meanwhile, 5-FU-β-cyclodextrin complex and 5-FU nanosponge exhibited fast loss after 230 °C.

The TGA thermograms indicated lower thermal stability upon the incorporation of 5-FU inside the 5-FU-β-cyclodextrin complex and 5-FU nanosponge. This difference can be attributed to the incorporation of 5-FU inside β-cyclodextrin polymer and nanosponge. A similar observation was reported in a previous study to encapsulate organic substances in cyclodextrin-based nanosponge (28).

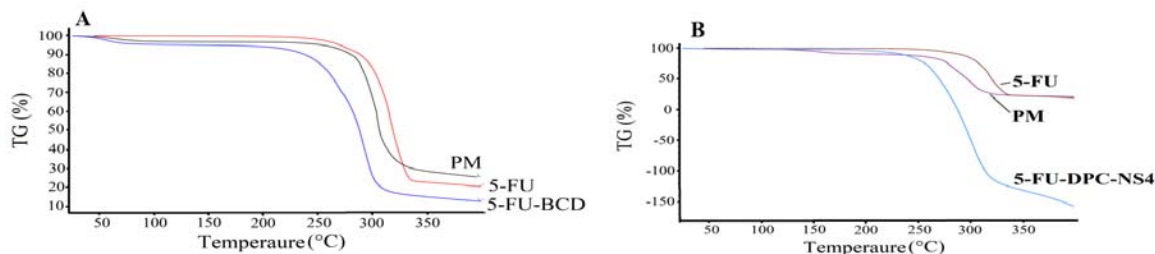


Fig. 5. Thermogravimetric analysis of 5-FU-cyclodextrin complex and the loaded DPC-NS4 compared to the raw drug and the corresponding physical mixture. FU, Fluorouracil; DPC, diphenyl carbonate; NS, nanosponge; PM, physical mixture.

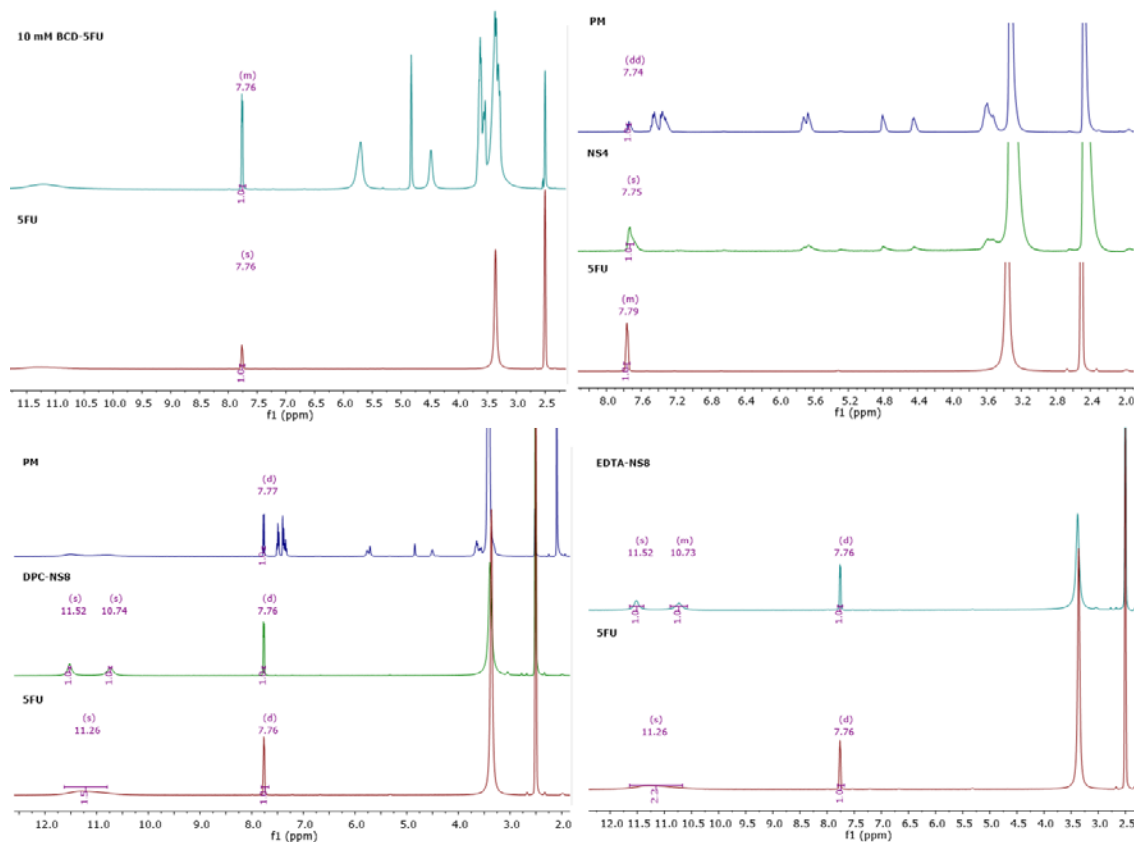


Fig. 6. 1H NMR spectra of (A) 5-FU-loaded 10 mM BCD, (B) 5-FU-loaded DPC-NS4, (C) 5-FU-loaded DPC-NS8, and (D) 5-FU-loaded EDTA-NS8, at 400 MHz, DMSO-d₆. BCD, β-cyclodextrin; FU, fluorouracil; PM, physical mixture; DPC, diphenyl carbonate; EDTA, ethylenediaminetetraacetic acid; NS, nanosponge.

1H NMR spectroscopy

¹H NMR spectra of 5-FU, 5-FU- β -cyclodextrin complex, 5-FU-loaded nanosponges DPC-NS4, DPC-NS8, and EDTA-NS8 are displayed in Fig. 6. Except for the DPC-NS4, all the recorded NMR spectra did not show any significant chemical shift changes from that of 5-FU indicating no complexation with 5-FU. In a recently published study by Melnikova *et al.*, no evidence of complexation was observed between 5-FU and β -cyclodextrin using ¹H NMR data (29).

Surface charge

The zeta potentials of the raw materials and the prepared samples are shown in Table 1. All raw materials and samples have negative zeta potential values without any significant difference from the reported values. On the other hand, the resulting two peaks for the zeta potential values of 5-FU loaded nanosponge EDTA NS8 in comparison to plain nanosponge and the raw drug, can be attributed to the presence of an unincorporated excess amount of free 5-FU.

Saturation solubility study

As represented in Fig. 7, no significant variations in the saturation solubility (24 h) of 5-FU complexed with β -cyclodextrin, DPC

crosslinked nanosponges in purified water, as compared to the raw 5-FU saturation solubility (11.74 ± 0.24 mg/mL). EDTA NS8 significantly enhanced 5-FU solubility to 12.94 ± 0.28 mg/mL, which is equivalent to a 10.22% increase, compared to the raw 5-FU. This increase in solubility can be attributed to the hydrophilic nature of the formed inter cavities that are formed when EDTA dianhydride is used as a crosslinker.

In vitro release experiments

Dissolution profiles of 5-FU in comparison with the prepared samples are represented in Fig. 8. The drug release experiments results did not show significant changes in 5-FU release between the prepared nanosponges, 5-FU- β -cyclodextrin, and raw 5-FU. This can be attributed to the low stability of the 5-FU formed a complex with β -cyclodextrin polymer and the recently reported short lifetime of the formed complex (13.5 ms) (29). The initial rate of 5-FU release calculated in the first 30 min was 1.79 ± 0.13 , compared to 2.21 ± 0.087 , 1.84 ± 0.15 , 2.23 ± 0.1 , and 2.49 ± 0.2 for 5-FU- β -cyclodextrin complex, NS4, DPC-NS8, and EDTA-NS8, respectively. The fastest initial release of 5-FU from EDTA-NS8 can be attributed to the resultant increase in the solubility as reported in the solubility study.

Table 1. Surface charges of the raw materials and the prepared samples, and the percent of 5-FU content in the prepared samples. Data are presented as mean \pm SD.

ID	Zeta potential	Drug content (%)
5-FU	-16.4 ± 4.16	
Raw β -cyclodextrin	-29.1 ± 5.37	
β -Cyclodextrin complex	-16.6 ± 4.27	9.98 ± 3.42
Plain NS4	-18.4 ± 5.63	
5-FU-loaded NS4 DPC	-11.6 ± 3.59	64.05 ± 5.08
Plain NS8	-11.9 ± 4.32	
5-FU-loaded NS8 DPC	-24.3 ± 6.27	42.22 ± 4.71
Plain EDTA NS8	-10.2 ± 5.62	
5-FU-loaded EDTA NS8*	-33.4 ± 4.32 (55.3%) -17.9 ± 3.29 (44.7%)	63.46 ± 3.48

*This sample showed two sharp peaks for zeta potential and these values for the percent of the intensity of each peak. FU, Fluorouracil; DPC, diphenyl carbonate; EDTA, ethylenediaminetetraacetic acid; NS, nanosponge.

SEM of 5-FU nanosponges

The SEM images of 5-FU, 5-FU- β -cyclodextrin complex, 5-FU-loaded NS4, and 5-FU-loaded EDTA NS8 nanosponge are shown in Fig. 9. As shown from the images, the porous nature of the β -cyclodextrin complex and the prepared nanosponge was observed. The 5-FU- β -cyclodextrin complex showed

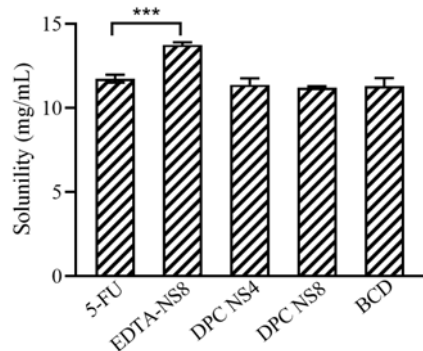


Fig. 7. Saturation solubilities of 5-FU, 5-FU- β -cyclodextrin complex, and 5-FU-loaded crosslinked nanosponges. FU, Fluorouracil; BCD, β -cyclodextrin; DPC, diphenyl carbonate; EDTA, ethylenediaminetetraacetic acid; NS, nanosponge.

dendrites in the images. The SEM images of the surface morphology for 5-FU-loaded NS4 showed irregularity in the sample with a clear presence of multilayers on the surface, while 5-FU-loaded EDTA NS8 nanosponge showed irregularity in the sample with the presence of an interesting flower-like multilayer structure for the prepared nanosponge.

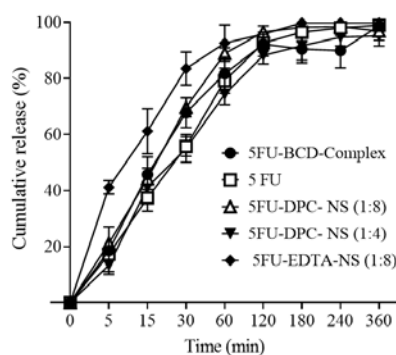


Fig. 8. *In vitro* release profiles of 5-FU, 5-FU- β -cyclodextrin complex, 5-FU-loaded EDTA, and DPC crosslinked nanosponges. FU, Fluorouracil; BCD, β -cyclodextrin; DPC, diphenyl carbonate; EDTA, ethylenediaminetetraacetic acid; NS, nanosponge.

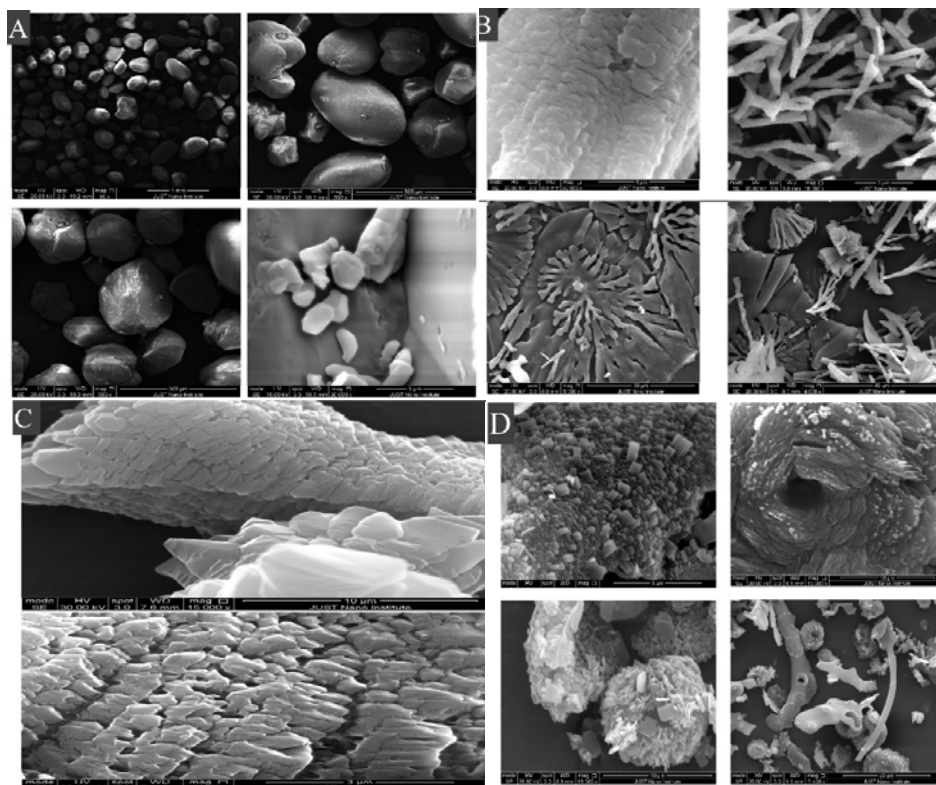


Fig. 9. SEM images of (A) 5-FU, (B) 5-FU- β -cyclodextrin complex, (C) 5-FU-loaded DPC crosslinked NS4 nanosponge and (D) 5-FU-loaded EDTA NS8 nanosponge with different magnifications. FU, Fluorouracil; DPC, diphenyl carbonate; EDTA, ethylenediaminetetraacetic acid.

The *in silico* design of 5-FU- β -cyclodextrin complexes

Preparation of the simulated 5-FU- β -cyclodextrin complexes 5-FU is a weak acid with a pKa value of 7.93-8.05 (30), which means it will be considerably ionized under physiological conditions. Hence, the neutral and ionized forms of 5-FU were prepared, using the Prepare Ligand protocol in Discovery Studio, and considered for *in silico* calculations. The top-scoring docked poses of the neutral and ionized 5-FU into β -cyclodextrin were selected as the starting structural models of the virtual complexes (Fig. 10). Fig. 10 shows that 5-FU is occupying the interior of β -cyclodextrin and is forming hydrogen bonds with polar regions in β -cyclodextrin. The neutral 5-FU forms three H-bonds, while the ionized one forms only one H-bond. Those two virtual complexes were then used to run MD simulations to investigate 5-FU- β -cyclodextrin complexation stability under aqueous conditions.

MD simulations of 5-FU- β CD complexes

The generated trajectories of the simulated complexes were analyzed by calculating thermodynamic properties (potential, kinetic, and total energy as well as temperature) and their root means square deviations (RMSD) (Fig. 11).

Figure 11 shows that the energies and temperature were stable throughout the simulation time. Regarding the RMSD values, there was a large increase in their values as the system started to flex relative to the starting minimized configuration until they plateaued around their mean values (17.10 Å for the neutral form and 17.15 Å for the ionized form) during the production phase. This indicates that something major has happened (could be an exclusion of the complexed 5-FU from BCD), therefore, we visually inspected the generated trajectories to investigate complexation stability.

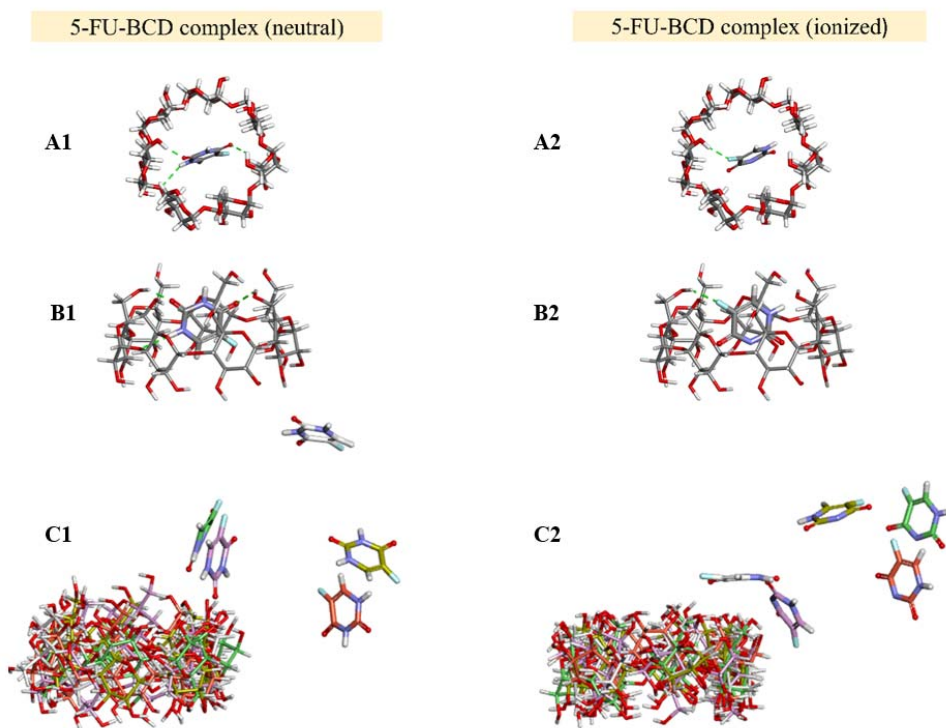


Fig. 10. Snapshots of 5-FU- β -cyclodextrin simulated systems at 2, 4, 6, 8, and 10 ns time intervals. (A) and (B) are top and side views of 5-FU- β -cyclodextrin starting structures (at 0 ns). (C) Side view of superimposed snapshots of the simulated complexes at different time points. H-bonds are shown as dashed green lines (when present).

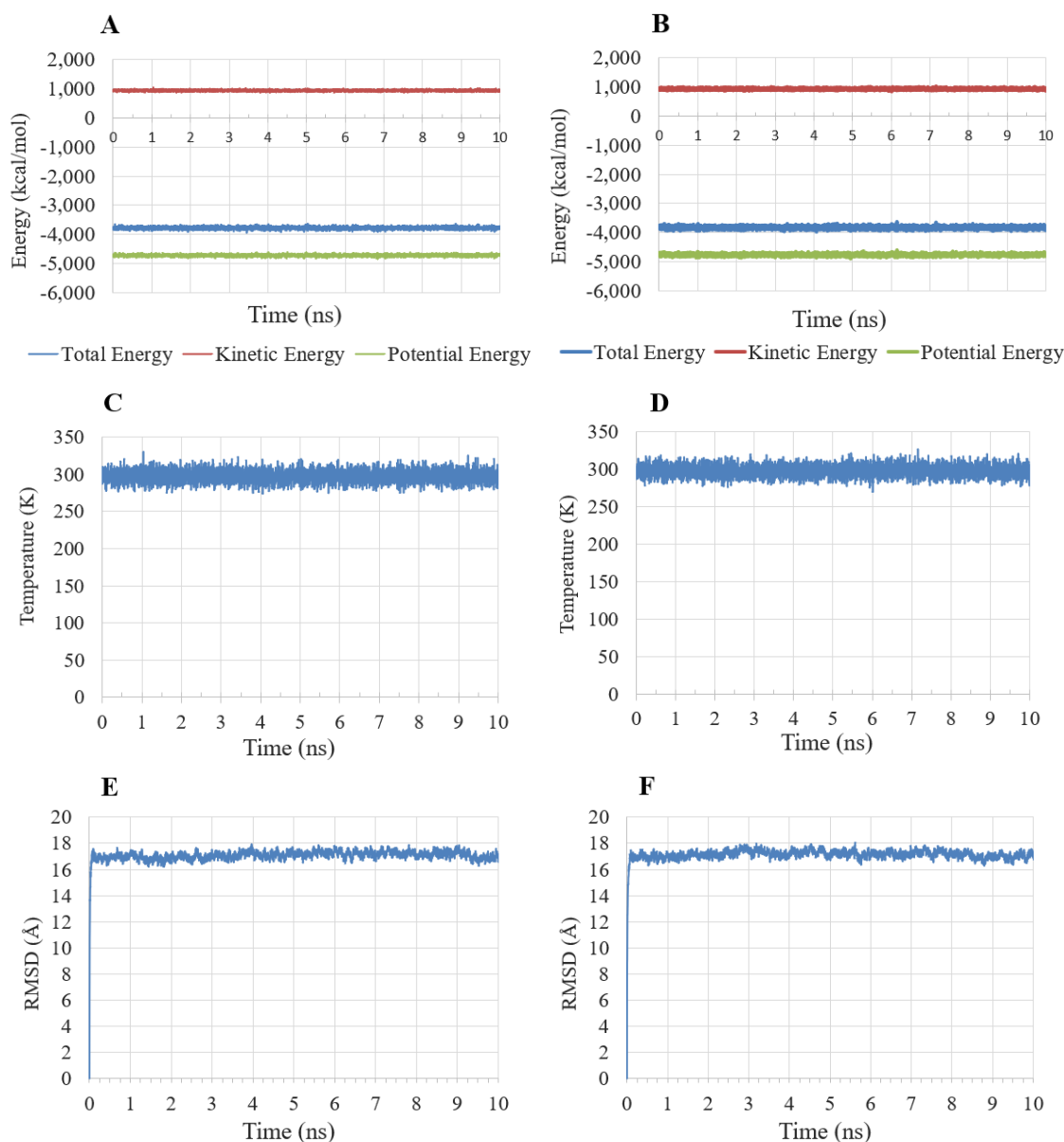


Fig. 11. Summary of the (A) energy, (C) temperature, and (E) RMSD changes during the simulation of the 5-FU- β -cyclodextrin complexes corresponds to the neutral 5-FU system. In parallel, the right panel corresponds to summary of the (B) energy, (D) temperature, and (F) RMSD changes during the simulation of the 5-FU- β -cyclodextrin complexes corresponds to the ionized system.

5-FU- β -cyclodextrin complexation stability

The simulation trajectories were visually inspected as they evolved with time, which provides a comprehensive perception of the complexation stability of simulated complexes. Snapshots taken from the two simulated systems at different time points (2, 4, 6, 8, and 10 ns) during their simulation course are shown in Fig. 10.

As shown in Fig. 10, 5-FU- β -cyclodextrin complexation was unsuccessful for both

simulated complexes. As soon as the MD production phase began, the 5-FU molecule had completely departed its host BCD and moved to the aqueous surroundings.

These results confirmed the predicted instability observed in the solubility and dissolution results. Such instability was observed by Melnikova *et al.* who reported short-term stability of the formed complex with a lifetime of 13.5 ms (29).

DISCUSSION

The complexation of 5-FU with cyclodextrin was previously reported in the literature (31). The stoichiometry of the inclusion complex was 1:1 for both α - and β -cyclodextrins. The formulated complexes enhanced the therapeutic efficacy of 5-FU by inhibiting the cell growth of different cell lines (31). In contrast, a recent study has shown the development of a 5-FU complex with cyclodextrin polymer in an aqueous solution and suggested the formation of an unstable inclusion complex (29). Moreover, a recent study suggested that cyclodextrin-based nanosponge formulations may be a promising technique for the delivery of 5-FU (32). This contradiction in the published studies necessitates the reconsideration of the complexation of 5-FU with cyclodextrin.

We concluded that in contradictory to the reported studies (33) we did not observe a clear indication of inclusion complex formation or enhancement in solubilization when 5-FU complexed with cyclodextrin.

The results of the PXRD analysis agree with the FTIR and DSC results suggesting the formation of the complex of 5-FU with cyclodextrin. On the other hand, MD simulation, saturation solubility, and the NMR results oppose the evidence of inclusion of complex formation, as the MD simulation indicated the high tendency of 5-FU to be departed from the cavity of cyclodextrin, and the NMR results did not show evidence of cross-linking, and the 5-FU- β -cyclodextrin complex did not show any increase in 5-FU solubility. This contradiction between solid powder characterizations and analysis in solvent media indicates the formation of the complex with low stability, such that when dissolved in water or NMR solvent, it has a substantial potential to re-separate.

Our study results indicated that 5-FU was not achieved the expected encapsulation with carbonate nanosponge in contradictory to the reported studies (32,34), this encouraged us to study the complexation of 5FU with the raw cyclodextrin to further explain the results. We also conducted preliminary phase solubility studies for both types of nanosponges and non-crosslinked cyclodextrin, all systems did not show changes in saturation solubility of 5-FU with increasing the level of cyclodextrin or the

nanosponges, so we fully characterized and evaluated the complex and samples to clarify the results.

Even though the thermal analysis results indicated the fusion between β -cyclodextrin-based nanosponge and 5-FU.

The appearance of most of the 5-FU characteristic peaks in the PXRD patterns suggested the existence of 5-FU on the surface of the cyclodextrin-based nanosponge rather than inside its cavity. In agreement with PXRD results, FTIR analysis of nanosponge cross-linked with DPC (NS4 and NS8) suggested the interaction between 5-FU and the cyclodextrin-based nanosponge NS4 rather than NS8, indicating a decrease in the crosslinked cyclodextrin nanosponge's ability to interact with 5-FU as the molar ratio of the DPC crosslinker increased, which can be attributed to the presence of an extra amount of the crosslinker that sterically hinders the entrance of 5-FU to their binding sites.

On the other hand, the study results indicated the influence of the cross-linking agent type on the resulting nanosponge, as the FTIR analysis of the 5-FU-loaded EDTA cross-linked nanosponge (EDTA NS8) suggested the interaction and loading of 5-FU with EDTA NS8. Additionally, cross-linking of β -cyclodextrin with an EDTA dianhydride cross-linker changed the physical properties of the formulated nanosponge, as the resulting nanosponge showed an amorphous nature of the plain unloaded nanosponge as confirmed by the PXRD, DSC, and SEM analysis.

The experimental results demonstrated no significant changes between 5-FU and 5-FU-cyclodextrin complex release profiles, indicating poor 5-FU-cyclodextrin complexation and instability in aqueous solutions. This statement was supported by MD simulations of 5-FU- cyclodextrin complexes.

EDTA-crosslinked nanosponge increased 5-FU saturation solubility and had a higher initial rate of drug release than cyclodextrin polymer or DPC-crosslinked nanosponge. This could be attributed to the hydrophilic nature of the EDTA crosslinker and the amorphous structure of the resulting nanosponge.

Our study results indicated the demand for further modifications of the nanosponge system to achieve the desired formulation characteristics and enhance stability.

CONCLUSIONS

Physicochemical characterization of dried formulations indicated the complexation of 5-FU with β -cyclodextrin polymer. Despite that, no clear evidence of 5-FU encapsulation was evident in the prepared β -cyclodextrin-based nanosponge. An *in vitro* release study revealed no significant differences in 5-FU, β -cyclodextrin complex, and β -cyclodextrin-based nanosponge release profiles, suggesting weak complexation and instability in aqueous solutions. Moreover, MD simulations have revealed the complexation profiles of 5-FU- β -cyclodextrin complexes at the atomic level and have proved that 5-FU- β -cyclodextrin complexation is unstable in agreement with the experimental results. Crosslinking of β -cyclodextrin with EDTA dianhydride crosslinker showed an enhancement in 5-FU saturation solubility combined with a faster initial rate of drug release. Suggesting that the use of hydrophilic EDTA crosslinker is more promising for the formulation of 5-FU-loaded nanosponge compared to DPC crosslinker. However, this formulation still requires further modifications to achieve the better size and textural properties and improve the 5-FU release properties.

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Authors' contributions

R. Obaidat and H. Mashaqbeh conceptualized the study. R. Obaidat supervised the study. H. Mashaqbeh conducted the experiments. H. Mashaqbeh and S. Alnabulsi contributed to the methodology. R. Obaidat, H. Mashaqbeh, N.A. Al-Shar'f, T. El-Elimat analyzed the data.

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