Synthesis and Evaluation of β-cyclodextrin Based Nanosponges of 5-Fluorouracil Using Ultrasound Assisted Method.

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Abstract

5-Fluorouracil (5-FU) is mostly used in the treatment of stomach cancer. After intravenous injection of 5-FU, it is rapidly distributed and eliminated with an apparent terminal half-life of 8-20 min. It is poorly absorbed after oral administration with an extremely variable bioavailability. Hence, this study has been made to synthesize 5-FU nanosponges (NS) to increase its accumulation in gastric tumors by the help of an enhanced permeability retention effect (EPR) and decrease its systemic side effects. On the other hand, 5-FU is sparingly soluble in water so its dissolution can be increased by incorporation in nanosponges as nanoparticles.

CD-nanosponges were prepared by crosslinking β-CD with diphenylcarbonate (DPC) using ultrasound assisted technique. 5-FU was incorporated with NS by freeze drying, and the phase solubility study, complexation efficiency (CE) entrapment efficiency were performed. Also, the particle morphology was studied using SEM and AFM. The in vitro release of 5-FU from the prepared nanosponges was carried out in 0.1N HCl.

5-FU nanosponges particle size was in the nano size. The optimum formula showed a particle size of (405.46±30) nm, with a polydispersity index (PDI) (0.328±0.002) and a negative zeta potential (-18.75±1.8). Also the drug entrapment efficiency varied with the CD: DPC molar ratio from 15.6 % to 30%. The SEM and AFM showed crystalline and porous nature of the nanosponges. In the in vitro drug release study of the selected formula 5-FUNS2 exhibited the fastest dissolution rate which is 56% in the first hr.

Different molar ratios of (cyclodextrin to crosslinker) (CD: DPC) has a proficient effect on complexation efficiency (CE), apparent stability constant (Kst) and entrapment efficiency of 5-FU. 5-FUNS2 with (1:4) molar ratio showed the best result of CE, Kst and entrapment efficiency. 5-FUNS2 gave a higher release rate than the 5-FU-βCD inclusion complex and 5-FU solution. Surface morphology of the prepared nanosponges by SEM, AFM indicate that nanosized and highly porous nanosponges was obtained. The overall results suggest that cyclodextrin nanosponges could be a promising 5-FU delivery system utilizing the suitable formula.

Keywords: 5-FU, nanosponges, ultrasound assisted method, βCD, DPC, SEM, AFM.

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Introduction

Although therapeutic agents can reduce tumor size and cancer remission and have a high potential to destroy cancer cells, they are not organ specific and can damage proliferative cells (1). One of the major goals of cancer therapeutics is to kill cancer cells without damaging normal tissues. One way to achieve this is the use of molecularly targeted therapy combined with chemotherapy. Tissue and cell distribution of cancer therapeutic drugs can be controlled by the entrapment in sub-micron level (<1 µm) colloidal systems, in other words known as nanoparticles. Some of the desirable characteristics that are needed to deliver therapeutic agents to tumor cells include the ability to overcome drug resistance at the tumor and cellular levels and ensure an appropriate distribution, biotransformation, and clearance of the drug (2).

Conventional chemotherapeutic agents work by destroying rapidly dividing cells. This is the main property of neoplastic cells which is why chemotherapy also damages normal healthy cells that divide rapidly such as cells in the bone marrow, macrophages, digestive tract, and hair follicles (3).

Nanosponges are hyper-cross-linked cycloextrinsics that can be obtained with α, β and γ cycloextrins, either alone or as mixtures containing relevant amounts of linear dextrin, cross-linked with a suitable cross-linking molar ratio, by using an active carbonyl compound, e.g., diphenyl carbonate, by ultrasound-assisted synthesis. Thus, spherical nanosponges of submicron size of cycloextrin are connected by nanochannels to form a cage-like structure. These nanosponges can be inclusion complex drug carriers (4).

Nanotechnology have been applied to improve drug delivery and to overcome some of the problems of drug delivery for cancer treatment (5). Nanosponges are a novel class of hyper-cross linked polymer based colloidal structures consisting of solid nanoparticles with colloidal and nanosized cavities. Nanosponges solubilizes poorly water soluble drugs and provides a prolong release as well as improves the drug bioavailability by modifying the pharmacokinetic parameters of active constituents (6, 7).

5-Fluorouracil (5-FU) was most commonly used in the treatment of cancers of colon, breast, stomach and pancreas (8). However, like other drugs used for chemotherapy, it affects the growth of normal body cells and often causes side effects such as hair loss, fatigue, birth defects, mouth sores, liver disease, and a temporary drop in bone marrow function (9).

After intravenous injection of 5-FU, it is rapidly distributed and eliminated with an apparent terminal half-life of 8-20 min with a pKa of 8, and 13, LogP(1) (10). Also 5-FU is poorly absorbed after oral administration with an extremely variable bioavailability (11). The aim of the study was synthesis and evaluation of 5-FU loaded nanosponges to enhance the dissolution rate of the sparingly soluble 5-FU. Also nanosponge increases its accumulation in gastric tumors by the help of an enhanced permeability retention effect (EPR) and decrease its systemic side effects. As it is intended to be formulated as floating tablet for local gastric cancer therapy in the future study.

Materials and Methods

Materials

5-Fluorouracil, βCD and diphenyl carbonate (DPC) were obtained from Hyper-chem Ltd Co. (Hangzhou, China). All other analytical reagents were of analytical grade.

Methods

Preparation of β-CD-nanosponges using ultrasound assisted method

Accurate amounts of βCD and diphenyl carbonate DPC were mixed in 100ml beaker at a different molar ratio as shown in Table (1). The beaker was then placed in an oil bath and heated to 90°C. Then the mixture was sonicated for 4 hours at 50% amplitude using ultrasound probe capable of supplying maximum power of 500 Watt at 20 kHz (Qsonica, USA). The reaction mixture is left to cool and the product obtained is broken up roughly. Numerous needle-shaped crystals of phenols can be seen on the clear surface of the beaker as shown in Figure (1) and part of the phenol developed contributes to agglomerating of the product (12).
Subsequently after cooling, the product was broken up roughly by mortar and repeatedly washed with an excess amount of distilled water (DW) through filtration by the Buchner funnel to remove unreacted βCD. An additional purification step consists of Soxhlet extraction in ethanol, which was performed for 24 hours to remove the unreacted DPC and phenol present as by-product of the reaction. Finally, the nanosponges (NS) were dried at room temperature to obtain a fine white powder.

Preparation of 5-FU inclusion complexes

One formula of β-cyclodextrin inclusion complex with 5-FU (5-FU–βCD) was prepared. A weighted amount of 5-FU was finely suspended in a water solution containing an equimolar amount of βCD and 5-FU (1:1). The aqueous suspension was then stirred at room temperature in the dark place for 24 h. After centrifugation (5000 rpm, 10 min), (Labent, Germany) the supernatant was freeze-dried.

Production yield of the prepared nanosponges

Production yield: The production yield can be determined by calculating initial weight of raw materials and final weight of nanosponges obtained.

Production yield: Practical mass of nanosponges = Theoretical mass of nanosponges (polymer + crosslinker) × 100

Encapsulation efficiency

Weighed amount of 5-FU-loaded nanosponges were dispersed in DW and sonicated for 10 min, then centrifuged at 15,000 rpm for 15 min (Copley, Germany) double cycle after that the supernatant was withdrawn, suitably diluted with distilled water and were subjected to UV spectroscopy for measuring the absorbance of the sample at the λmax of 5-FU (266 nm). With the help of absorbance, the concentration in the supernatant was determined by plotting the absorbance value against concentration in the standard curve.

Phase solubility studies

Phase solubility studies were carried out according to the Higuchi–Connors method. An accurate amount of 5-FU (100 mg) was added to a series of aqueous solutions (5 mL) containing increasing concentrations of βCD-NS, from (9.5 to 12.7) mM and in βCD (1:1). The samples were stirred in the dark at room temperature for 5 days. After equilibration, the aqueous suspensions were centrifuged and the 5-FU content in the supernatant was determined by UV spectrophotometer at 266 nm.

Figure 1. a) Structural representation of reaction for preparation of NS, b) Ultrasound assisted method

Table 1. The composition and molar ratios of CD:

| Formula codes** | β-CD:DPC* Molar ratio | β-CD (g) | DPC (g) |
|-----------------|------------------------|----------|---------|
| NS 1            | 1:2                    | 2.27     | 0.856   |
| NS 2            | 1:4                    | 2.27     | 1.713   |
| NS 3            | 1:6                    | 2.27     | 2.57    |
| NS 4            | 1:8                    | 2.27     | 3.427   |
| NS 5            | 1:10                   | 2.27     | 4.28    |

DPC used to prepare βCD-NS*.

* β-CD β-cyclodextrin, DPC Diphenyl carbonate. ** 1 mole of β-CD = 1.135 g, 1 mole of DPC = 0.2142 g. *** NS1-NS5 plain NS
The phase solubility diagram was constructed by plotting the total molar concentration of 5-FU against the molar concentration of βCD-NS. Stability constants (Kst) from the phase solubility diagram were calculated using the Equation (1):

$$Kst = \frac{\text{slope}}{S_0(1-\text{slope})}$$

(1)

Where, \(S_0\) represents the solubility of 5-FU in the absence of CD. The slope was determined from the initial linear part of the concentration curves of 5-FU.

The complexation efficiency (CE) is the concentration ratio between cyclodextrin in a complex and free cyclodextrin, and it was calculated from the phase-solubility diagrams (15).

The complexation efficiency is calculated by the slope of the phase-solubility profile using equation 2, which is referred to as the complexation efficiency (CE) (23).

$$CE = S_0K1:1 = \frac{D_{\text{CD}}}{\text{CD}} = \frac{\text{slope}}{1 - \text{slope}}$$

Since, the numerical value of CE is only dependent on the slope of the phase-solubility profile, less variation is usually observed in the CE values compared to the stability constant Kst value.

Characterization of the prepared 5-FU loaded nanosponges

Particle size, Polydispersity Index analysis (Dynamic light Scattering ) and zeta potential Nanosponges sizes and polydispersity index were measured by dynamic light scattering using a 90 plus particle sizer (ZetaPlus Particle Sizing, NY, Software, Version 5.34). The samples were suitably diluted with water prior to measurements. Zeta potential measurements were also made using an additional electrode in same instruments.

The mean hydrodynamic diameter (Dh) and polydispersity index (PI) of the particles were calculated in intensity using the cumulant analysis after averaging the three measurements (17, 24).

Fourier Transform-Infrared Spectroscopy (FT-IR)

ATR-FTIR spectra of 5-FU, DPC, βCD, βCDNS and 5-FUNS were recorded on a IRAffinity-S1 Spectrum FT-IR (Shimadzu, Japan) in the region of 4000–650 cm⁻¹. It was performed, using a Shimadzu spectrophotometer, to confirm the formation of βCDNS and understand if there are interaction between drug and NS (15).

Scanning Electron Microscopy (SEM)

Scanning electron microscopy (Tescan Mira3,France) was significant for determination of surface characteristics and size of the particle. Scanning electron microscope was operated at an acceleration voltage of 15 kV (25).

Atomic Force Microscopy (AFM)

A further in-depth morphological analysis was performed using an atomic force microscope (Angstrom Advanced Inc. AA3000) with a scanner of 3.1 μm with three piezo electrodes for three axes X, Y and Z in a noncontact mode. The sample suspensions (1% w/v) were prepared in distilled water and a drop was impregnated onto aluminum sheet (2 cm×2 cm). This was allowed to dry in a HEPA filter zone and the dried region was analyzed (36).

In-vitro release study of 5-FU nanosponges

In-vitro release study of 5-FU from 5-FU-βCD inclusion complex and the selected formula of 5-FUNS was performed by using an accurate amount of impregnated nanosponges equivalent to 100 mg 5-FU suspended in 5 ml of 0.1N HCl solution which was placed in the dialysis membrane (cut off 12,000 Da) and the samples were individually placed in dissolution vessel containing 900 ml of 0.1N HCl, maintained at 37 ± 0.5°C at 75 rpm using a paddle dissolution apparatus (USP Type II). At various time intervals, aliquots of 5ml were withdrawn and replaced with the same volume of fresh dissolution medium to maintain the sink conditions and the withdrawn samples were analyzed by UV spectrophotometer (EMCLAB, Germany) at 266 nm (27, 28).

Statistical analysis

The results are reported as the mean±SD and statistical significance was determined using one-way analysis of variance (ANOVA) and Student’s t-tests as appropriate. All experiments were performed in triplicate and values were expressed as the mean standard deviation SD. Values of \(p < 0.05\) were considered statistically significant (29).

Results and Discussion

Production yield

The practical yield of nanosponges was found to be less for lower (β-CD: DPC) molar ratio (1:2). The practical yield increased with the increase in molar ratio up to 1:8, and at higher molar ratios (1:8 - 1:10), the yield was found to be almost the same for both molar ratio. This may be due to saturation of the reactive functional groups at higher concentration (30) the molar ratio significantly (\(p<0.05\)) affected the production yield of CD NS.
Table 2. Production yield percent of the prepared CD NS, data are expressed as Mean ± SD, n = 3, standard deviation (SD).

| Formulas No.* | Theoretical weight (g) | Production yield (g)±SD | Production yield % |
|---------------|------------------------|-------------------------|--------------------|
| NS 1          | 2.702                  | 1.85±0.19               | 48.1               |
| NS 2          | 3.13                   | 3.18±0.3                | 63.9               |
| NS 3          | 3.559                  | 4±0.1                   | 70.2               |
| NS 4          | 3.987                  | 4.72±0.2                | 72.7               |
| NS 5          | 4.414                  | 5.64±0.3                | 74.8               |

*NS1-NS5 = plain nanosponges.

Phase solubility study

Phase solubility studies were conducted for all the prepared nanosponges and their respective native β-cyclodextrin in DW. The solubility of 5-FU was found to be about (0.02 M) in the absence of β-cyclodextrins nanosponges. Also, the molar concentration of NS was determined by calculating the molecular weight of NS according to the chemical formula that was mentioned in Figure (1). The phase solubility diagram revealed that the solubility of 5-FU increased linearly as a function of increasing cyclodextrin nanosponges concentration indicating the phase solubility profile obtained was an “A-type” diagram, according to the Higuchi and Connors classification (22).

Cyclodextrin-based nanosponges showed superior complexing ability than natural cyclodextrins towards many molecules. The parent βCD complex shows lower complexation efficiency (CE) and apparent stability constant (Kst) (0.22) and (10±0.5 M⁻¹) respectively, as shown in Table (3). These values were lower than that obtained by βCD nanosponges. This gave significant (p<0.05) differences of CE and Kst values between the prepared CDNS and the parent CD. This is due to the carbonate linkage which was added to the primary hydroxyl groups of the parent βCD unit. Thus, the drug molecules could be included inside the nanocavities of βCD and due to the cross-linking further interactions of the guest molecules with more βCD units might be thought. Moreover, the presence of the cross-linked network might also form nanochannels in the NS structure for the polymer mesh (16). Figures (2a) and (2b) shows the phase solubility diagram of 5-FU with the prepared NS and βCD, respectively. The slopes of the curves of complexes were lower than one, demonstrating the formation of 1:1 inclusion complex.

Table 3. Different parameters of phase-solubility studies of 5-FU with the prepared NS and βCD in distilled water, at 25 °C.

| Formulas codes | Slope (M⁻¹) | Intercept Sₒ (M) (5-FU solubility) | R² | Kst (M⁻¹) mean ± SD* | Complexation efficiency (CE) |
|----------------|-------------|----------------------------------|----|----------------------|-----------------------------|
| 5-FUN S 1      | 0.3856      | 0.02                             | 0.936 | 30.2±1.6             | 0.63                        |
| 5-FUN S 2      | 0.6511      | 0.02                             | 0.9817 | 89.5±3.6             | 1.87                        |
| 5-FUN S 3      | 0.5811      | 0.02                             | 0.8699 | 66.3±2.5             | 1.39                        |
| 5-FUN S 4      | 0.4502      | 0.0199                           | 0.9725 | 41.1±1.4             | 0.81                        |
| 5-FUN S 5      | 0.4358      | 0.02                             | 0.9702 | 37.2±3.1             | 0.77                        |
| 5-FU-βCD Complex | 0.18       | 0.02                             | 0.987 | 10.7±1.2             | 0.22                        |

*Data are expressed as mean ± SD, n =3, standard deviation (SD).
Encapsulation efficiency

Among different types of nanosponges, the encapsulation efficiency of β-CDNS for 5-FU was observed to be higher in 5-FUNS2 (β-CDNS\(_{1:4}\)) as much as 30±2.3% w/w followed by 5-FUNS3 (β-CDNS\(_{1:6}\)) 25±2% and NS5 (β-CDNS\(_{1:10}\)) 22±2% as shown in Table 4.

The results revealed that the degree of cross-linking affected the encapsulation efficiency of nanosponges with a significant difference (p<0.05) between 5-FUNS1 and 5-FUNS2. It was found that at 1:2 molar ratio, the degree of cross-linking may be low, resulting in insufficient nanochannels for the guest complexation; thus 5-FU might not be encapsulated in higher amounts.

Particle size, polydispersity index analysis and zeta potential

The dynamic light scattering (DLS) related measurements were carried out after lyophitization. Table 5 illustrates the particle size values of the prepared 5-FU nanosponges of βCD-NS in which the smallest value was (256.3±24nm) and the largest one was (846.8±51nm). The overall sizes of NS found in the submicron range (<1μm) might be due to charge accelerated aggregation and molecular nature of relative CDs, resulting in a size increment. The increased size may be due to the aggregation during the drying process (31).

Zeta potential predicts the long term stability of the nanosized formulations (32). Zeta potential as a measure of surface charge was tested for 5-FU nanoformulations that have small particle size and lower PDI (5-FUNS2 and 5-FUNS3). The results of zeta potential obtained are presented in Table 4.

Table 4. Characterization of 5-FU nanosponges, (mean±SD) n=3

| Formula code | CD:DPC | Particle size ± SD (nm)* | PDI | ZP (mV) | Encapsulation efficiency |
|--------------|--------|--------------------------|-----|--------|-------------------------|
| 5-FUNS 1     | 1:2    | 545.45±23                | 0.492±0.01 | -     | 15.6±2.6 |
| 5-FUNS 2     | 1:4    | 405.46±30                | 0.328±0.002 | -18.75±1.8 | 30±2.3 |
| 5-FUNS 3     | 1:6    | 435.43±18                | 0.464±0.02 | -16.1±1.2 | 25±2 |
| 5-FUNS 4     | 1:8    | 846.83±51                | 0.359±0.01 | -     | 19±1.2 |
| 5-FUNS 5     | 1:10   | 256.3±24                 | 1.711±0.1 | -     | 22±1.7 |

On the basis of particle size, polydispersity index, zeta potential and encapsulation efficiency formulas 5-FUNS2 was chosen as the optimized formula for the preparation of nanosponges.

Drug-exciipients compatibility studies

The spectrum of 5-FU shows characteristic absorption bands in the region between 1656 and 1723 cm\(^{-1}\) correlated to the C=C, C=N, C=O, while the region at 1247–1425 cm\(^{-1}\) was assigned at the vibration of the substituted pyrimidine. The bands at 470, 551, 642, 749, and 813 cm\(^{-1}\), as well as those between 2407 and 3100 cm\(^{-1}\) are due to the aromatic ring (33), as shown in Figure(3).

Figure 3. FTIR of 5-FU
The appearance of the new peak of the carbonyl (C=O) group at 1751 cm$^{-1}$ in NS spectra confirmed the successful cross-linking of relative βCDs by DPC in various ratios$^{(31)}$. The 5-FU characteristic peaks were broadened or shifted in the formulations suggesting definite interactions between 5-FU and NS$^{(16)}$.

The peaks correlated to the aromatic ring for the drug alone are weakened in the spectra of 5-FU loaded NS and some bands in the region between 2407 and 3100 cm$^{-1}$ correlated to the aromatic ring result disappeared. These changes suggest the formation of the inclusion complexes$^{(34)}$, as shown in Figure(7).

**Figure 4.** FTIR of di-phenyl carbonate

**Figure 5:** FTIR of β-cyclodextrin
Morphology studies
AFM has been employed to Figure molecular structure of β-CD NS in the distilled water and examine their mechanical assets. The spherical crystalline NS presented the spectacular crystal planes with ordinary height of less than 400 nm. The SEM images of the plain βCD nanosponges were shown in Figure 8. SEM analysis revealed that nanosized particles with numerous pores on its surface.
Drug release was performed in 0.1 N HCl. The 5-FU cumulative percent release of 5-FNS2 (Fig. 4a) showed a burst effect at the end of first hour. This fast release (56%) of the active ingredient was a result of increase in solubilization of the drug. After the first hour, the drug was released in a controlled manner indicating encapsulation of 5-FU in the nanostructures.

5-FU release from 5-FUNS2 was found to be higher than 5-FU-βCD complex(1:1) as compared to 5-FU solution as mentioned previously, this is belong to the carbonate linkage which was added to the primary hydroxyl groups of the parent CD unit. Thus, the drug molecules could be included inside the nanocavities of CD and due to the cross-linking further interactions of the guest molecules with more CD units might be thought. Moreover, the presence of the cross-linked network might also form nanochannels in the NS structure of the polymer mesh. This peculiar structural organization might be responsible for the increased solubilization and protection capacities of NS in comparison with the parent CD (16).

**Conclusion**

Different molar ratios of (cyclodextrin to crosslinker) have a proficient effect on CE, Kst and entrapment efficiency of 5-FU. 5-FUNS2 with (1:4) molar ratio shows the best result of CE, Kst and entrapment efficiency. 5-FUNS2 gave higher release rate than 5-FU-βCD inclusion complex and 5-FU solution.

Surface morphology of the prepared nanospheres by SEM, AFM and indicated nanosized and highly porous nanospheres. The overall results suggest that cyclodextrin nanosponge could be a promising 5-FU delivery system utilizing the suitable formula.

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