Cationic cyclodextrin/alginate chitosan *nanoflowers* as 5-fluorouracil drug delivery system

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ABSTRACT

Cyclodextrins (CDs) have widely been used as component of drug delivery systems. However unmodified cyclodextrins are associated with cytotoxicity and poor water solubility thus limiting their use in pharmaceutical industry. The cationic-β-cyclodextrin (Cat-β-CD) polymer cores were synthesized using β-CD, epichlorohydrin and choline chloride *via* a one-step polycondensation process. The main aim of this study was to synthesize hierarchical *nanoflowers* composed of cationic-β-CD as polymeric core along with alginate and chitosan "petals" (Cat-β-CD/Alg-Chi *nanoflowers*) as carriers for oral delivery of 5-Fluorouracil (5-FU) *via* an ionic-gelation technique. The drug loading capacity, particle size, zeta potential and surface morphology of the synthesized *nanoflowers* were determined. The prepared *nanoflowers* were formed with an average size of 300 nm and a zeta potential of $+9.90$ mV with good encapsulation efficiency of up to 77.3%. *In vitro* release of 5-FU from the loaded *nanoflowers* showed controlled and sustained release compared to the inclusion complex alone. Cat-β-CD/Alg-Chi *nanoflowers* were assessed against L929 cells and found to be effectively inhibiting the growth of L929 cells in a concentration dependent manner.

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1. Introduction

An antimetabolite, 5-Fluorouracil (5-FU), has been used as an anticancer agent against various cancers like those of the colon or rectum for many years [1–3]. The main challenge of using 5-FU as with many other chemotherapy agents is its short biological half-life, toxic side effects and death of normal healthy cells causing side effects such as hair loss, fatigue, ulcers, and liver disorders [4]. Out of all delivery system, the oral route still continues to be most viable pathway for administration of drugs, especially where numerous doses are obligatory because it is non-invasive, avoids injections and risks associated with intravenous or subcutaneous delivery, such as acute infection. It has been suggested that chitosan nanoparticles can be used an as effective delivery system to overcome the side effects caused by the 5-FU [5]. In a recent study, pH responsive, (Tripolyphosphate) TPP-crosslinked 5-FU encapsulated chitosan nanoparticles were evaluated and computational experiments were conducted to judge whether it could be used as drug delivery agents for anticancer therapy [6]. Dubey & Parikh, (2004) prepared

chitosan microspheres of 12 μm size loaded with 5-FU [7] whereas, B. Arica et al. developed alginate bead nanoparticles loaded with 5-FU for breast cancer [8]. A poly-(ε-caprolactone) delivery system for 5-FU has also been developed for anticancer therapy where binding of 5-FU onto poly-(butylcyanoacrylate) nanoparticles showed enhanced efficacy towards anticancer activity [9]. All the systems mentioned above seem to face disadvantages associated with low encapsulation of the drug or low stabilization of the nanoparticles. It seemed to us that there would be an advantage in exploiting the best aspects of these current systems in order to overcome some of the limitations.

To achieve oral drug delivery for 5-FU, a nano drug delivery system is required to have certain important properties including: a) substantial biocompatibility b) protection from enzymatic degradation c) reduced leakage of drug during transportation d) controlled release of drug using biodegradable and biocompatible polymers e) reduced toxicity and f) increased therapeutic efficiency of the drug with reduced dosage frequency [10–12]. Ideally, the development of a nanocarrier system with controlled release could enhance the therapeutic drug effect at the specific target site and reduced dosage frequency leading to a better patient compliance and shorter duration of treatment [13,48].

Cyclodextrins (CDs) have attracted a great deal of attention in the pharmaceutical industry not only for their structure but also for their unique characteristics. CDs are not easily hydrolyzed or absorbed in the stomach or intestine but can be fermented to small saccharides

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by colonic microflora [14]. These characteristics are attractive for an ideal specific carrier delivery system to the colon. CDs when conjugated with the drug can reach the colon in an intact form and will release the drug after enzymatic degradation by micro-organisms in the lower intestinal tract without getting absorbed in the stomach. Udo et al. (2010) synthesized 5-FU/β-CD conjugates through ester or amide bond linkages and evaluated the drug release behavior for colon specific delivery [15].

β-cyclodextrin (β-CD) is made up of 7 glucopyranose units with hydrophobic cavity interior and hydrophilic exterior. Cyclodextrins are known to form inclusion complexes with organic compounds thus changing the physicochemical properties such as solubility, stability and bioavailability by non-covalent interactions [16]. CDs can break down to smaller derivatives and can get easily absorbed in the large intestine making them an ideal carrier for oral administration of drugs [17]. However, unmodified cyclodextrins are associated with reduced solubility and some hepatotoxicity, which reduces their application in the pharmaceutical industry [18]. To overcome this problem various cyclodextrin derivatives like hydropropyl-β-cyclodextrin (2HPβ-CD) and sulfobutylether-β-cyclodextrin (SBEβ-CD) have been developed. Cationic cyclodextrins have gained more attention due to their high solubility and stability. One of the most prominent groups of cationic cyclodextrins used in this study is the polycationic derivative that can be synthesized using a one-step poly-condensation method [19]. This cyclodextrin derivative has greater solubility in water as compared to parent β-CD. The inimitable trait of this cationic cyclodextrin makes it an ideal host to perform dual function during synthesis. Firstly, it acts as a host to form an inclusion complex with 5-FU and secondly its polycationic nature promoted the formation of **nanoflowers** (by ionic gelation technique). Cationic cyclodextrin polymers have been reported to play a safe role of drug delivery for gene delivery [9]. This system has also been shown to be an ideal, controlled released system for protein delivery such as insulin, by forming a complex with cationic cyclodextrin and encapsulation of the complex within the polymers [20].

A polyelectrolyte complex formed by alginate and chitosan is very well known [21]. Alginate and chitosan have received great attention in the pharmaceutical field because of their biological properties such as biodegradability, biocompatibility and non-toxicity. Alginate is a hydrophilic polymer made up of D-mannuronic (M) and L–glucoronic acid (G) residues joined linearly by 1, 4-glycosidic linkages. It is extensively associated with gel-forming capacity [22]. Chitosan, a mucoadhesive polymer is randomly composed of β (1–4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D- glucosamine (acetylated unit). Alginate, when crosslinked with divalent cations and chitosan has been widely studied for biomedical applications [23,24]. A nanocarrier system using combination of chitosan and alginate has been used as a carrier of 5-FU in ophthalmic delivery [25–27]. A mucoadhesive characteristic of alginate and chitosan helps to maintain contact with the epithelium for a longer period of time thus giving scope for the drug to pass through intestinal membrane. For effective delivery of insulin and small molecule drugs, alginate/chitosan nanoparticles have been studied for oral absorption and oral bioactivity [28,49].

In this work, we propose the combined use of these two systems for the delivery of 5-FU thereby taking advantage of the properties of both cationic cyclodextrin and biodegradable polymers. This was achieved by firstly forming an inclusion complex of the drug with cationic cyclodextrin acting as a carrier for the drug and secondly by linking the cationic cyclodextrin with polyanionic polymer, alginate. We report for the first time, an oral formulation of Cat-β-CD/5-FU loaded alginate/chitosan **nanoflowers**. Polyelectrolyte complexes and Cat-β-CD play a vital role by retaining the drug within the core of alginate/chitosan **nanoflowers**. This may certainly lead to a decrease in the degradation of the drug in the gastric environment avoiding the toxicity caused by the drug along with the enhancement of permeability of drug.

2. Material and materials

2.1. Materials

β-cyclodextrin (β-CD) was obtained from Walker Chemie (Munich, Germany). Epichlorohydrin (EP), Choline chloride (CC), 5-Fluorouracil (5-FU), deuterium oxide (99%, purity), low viscosity sodium alginate and low molecular weight chitosan (molecular weight 7464 g/mol) were purchased from Sigma-Aldrich (South Africa). Hydrochloric acid, acetic acid and sodium hydroxide were purchased from Merck chemical company. Deionized water was used for all the experiments. All other reagents were of analytical grade and were used without further purification.

2.2. Preparation of cationic-β-cyclodextrin polymer (Cat-β-CD)

Cationic cyclodextrin (Cat-β-CD) was synthesized following a procedure reported earlier [19] with slight modifications. In this study, the molar ratio of β-CD/EP/CC was selected to be 1:15:1, respectively. A typical polymerization reaction of β-CD for molar ratio β-CD/EP/CC is as follows: Sodium hydroxide, (NaOH) (1 g) was dissolved in water (20 mL), and then β-CD (5.675 g) was dissolved in the sodium hydroxide solution. The resulting solution was magnetically stirred at room temperature for 24 h. Thereafter, choline chloride (0.698 g) was fed into the solution rapidly and epichlorohydrin (6.940 g) was added dropwise at a flow rate of 0.1 mL/min. After completion of EP addition, the mixture was heated at 60 °C under stirring at 600 rpm for 2 h. The polymerization was terminated by neutralizing with aqueous hydrochloric acid solution (3 N). The solution obtained was dialyzed for 24 h with a dialysis membrane (Nominal Molecular weight-cut off 2000) to remove unreacted EP and CC. The solution obtained was evaporated and the solid pulverized to powder (Fig. 1a).

2.3. Preparation of Cat-β-CD/5-FU inclusion complex

The inclusion complex of Cat-β-CD with 5-FU was prepared using lyophilisation method as follows. An accurately weighed amount of Cat-β-CD (1.135 g) was placed into 100 mL round bottom flask and 50 mL of deionized water was added. The mixture was then stirred until dissolved. 5-FU (0.1308 g) was dissolved in deionized water (50 mL), slowly added (dropwise) into the Cat-β-CD solution and stirred for about 24 h at room temperature. After stirring, a nearly clear solution formed that was lyophilized using a freeze dryer (VirTis BenchTop K) yielding cyclodextrin inclusion complex as white powder. This material was assayed spectrophotometrically to determine the drug content included. The inclusion complex was characterized by FTIR, DSC, ^{1}HNMR, 2D-NMR and SEM.

2.4. Preparation of Cat-β-CD/5-FU loaded Alg/Chi nanoflowers

The Alg/Chi **nanoflowers** were spontaneously obtained using method modified described earlier [29]. Briefly, sodium alginate was dissolved in deionized water under continuous stirring overnight. Upon ensuring that all alginate powder was completely dissolved, the resulting solution (0.063 w/w %) (19.5 mL) was then mixed with the inclusion complex (3 mL of 3.5 mg/mL) under magnetic stirring for 30 min. CaCl$_2$ (1.3 mL of 18 mM) was then slowly added (dropwise) under constant magnetic stirring to the alginate solution containing the inclusion complex to provide pre-gel. Subsequently different concentrations of aqueous chitosan solutions (0.05–0.08% w/w) (4.2 mL) were added to the calcium alginate pre gel and stirred for 30 min. Chitosan was previously dissolved in 1% acetic acid under magnetic stirring for 24 h. The resulting **nanoflowers** were collected by centrifugation (10,000g, 4 °C) for 30 min using a Merck Eppendorf 5403R centrifuge. It is being represented schematically in Fig. 1b.
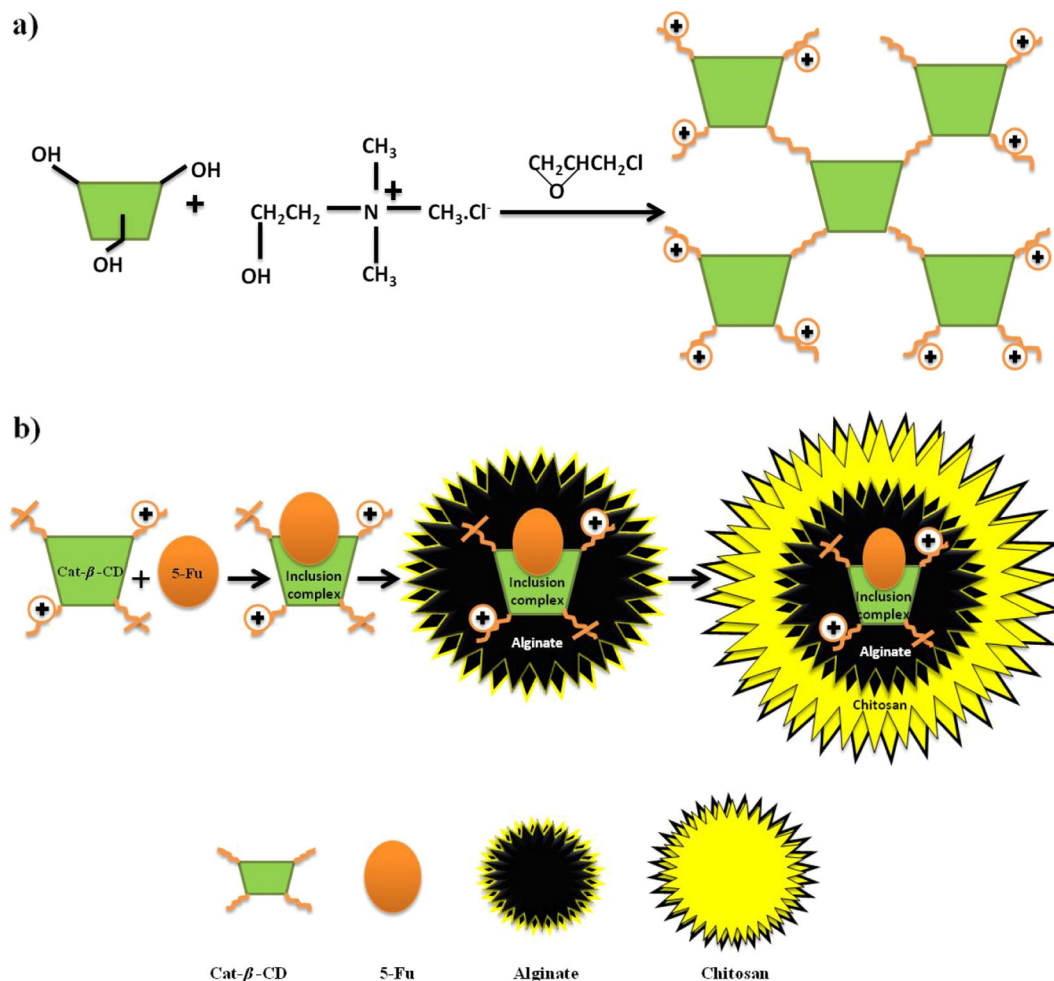
Fig. 1. Schematic illustration of a) polymerization from native β-CD to Cat-β-CD in a molar ratio of β-CD/EP/CC (1:15:1) b) representation of the inclusion complex (*Cat-β-CD/5-FU)* formation and its encapsulation within Alg/Chi **nanoflowers.**

2.5. Characterization

2.5.1. Fourier transform infrared spectroscopy (FTIR)

FTIR spectra were measured on a Perkin Elmer 100 FTIR spectropho-tometer with 4 cm^{-1} resolution averaging 12 scans in the range 3700 to 600 cm^{-1} in attenuated total reflectance (ATR) mode.

2.5.2. Nuclear magnetic resonance (^{1}HNMR and 2D-NMR)

All ^{1}H NMR, HMBC, HSQC, and NOESY spectra were recorded on a 9.4 T Bruker AVANCE III 400 MHz instrument at room temperature (298 K) with DMSO as an internal standard using a sample concentra-tion in the range of 10 (mg/mL). The spectrometer was equipped with a BBO probe with a z gradient. The data was recorded and analysed with Topspin 2.3 (Bruker, Karlsruhe, Germany) software. Standard Bruker pulse sequences were used: 1D ^{1}H (16 scans) and ^{13}C with ^{1}H decoupling and 2048 scans. For 1H —^{1}H NOESY experiment the mixing time was recorded with 50 mins ^{1}H and ^{13}C spectral width was 20.54 and 238 ppm, respectively.

2.5.3. Zeta size and zeta potential

Particle size distribution and zeta potential of colloidal system was obtained through dynamic light scattering (DLS) and laser Doppler an-emometry respectively using Malvern Zetasizer Nano model ZEN 3600 (Malvern instrument, Malvern, United Kingdom). The analysis was per-formed in triplicate at 25 °C.

2.5.4. Particle morphology

The morphology of the drug, cationic cyclodextrin and inclusion complex along with the morphology and surface structure of the nano-particles were examined using scanning electron microscopy, Tescan Vega operated at 20 kV. Samples were examined to characterize the changes of the drug surface before and after complexation process for indication of complex formation. The lyophilized nanoparticles were mounted on metal stubs, carbon coated under vacuum and then analysed.

2.5.5. Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM, JOEL, JEM-2100F, 200 kV) was used to observe morphology of the **nanoflowers**. In order to per-form observations, a drop of **nanoflowers** suspension was deposited onto a copper grid and dried at room temperature before viewing by TEM.

2.5.6. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) curves were recorded using a thermal analyzer TA instruments, model Q600 SDT. DSC was used to analyze the thermal behavior of the model drug 5-fluorouracil, cation-ic-β-CD, and the inclusion complex. The samples were heated in a sealed aluminium pan at a rate of 10 °C/min from 30 to 800 °C under constant purging of nitrogen atmosphere at 20 mL/min. The sample was weighed and placed in an aluminium pan and an empty aluminium pan was used as reference.

2.6. Entrapment efficiency of 5-FU into Alg/Chi nanoflowers

The entrapment efficiency (EE) of 5-FU encapsulated into the nanoparticles was determined using UV–Vis analysis (λ = 265 nm, Shimadzu, UV-2450) spectrophotometrically. The non-associated drug was isolated from the nanoparticles by centrifugation technique. The supernatant was assayed for the unbound drug content. The remaining nanoparticles were freeze dried at −40 °C for 24 h. Entrapped drug quantity is the total drug quantity determined in **nanoflowers** after the elimination of unbound drug by centrifugation.

Encapsulation Efficiency (EE) is calculated using the following formula [30].

$$\% \text{ Encapsulation efficiency} = \frac{\text{Total amount of 5FU-Free 5FU}}{\text{Total amount of 5FU}} \times 100$$

2.7. In vitro release of 5-FU from the nanoflowers

In vitro drug release behavior was studied in phosphate buffer saline solution at two different pH values (pH 2.3 and pH 7.4 respectively) using the dialysis technique. According to this method, freeze dried nanoparticles (5 mg/mL) were placed into the dialysis bag (MWCO 2KDa) which was transferred in a 50 mL phosphate buffer solutions (pH 2.3 and 7.4, respectively). The system was maintained at 37 °C in a water bath under constant stirring. The receptor compartment was sealed during the experiment to avoid evaporation of the receptor phase. At pre-determined intervals, an aliquot (1 mL) of dissolution medium was withdrawn and assayed for released drug content. The volume of each aliquot removed was compensated by the same volume of the dissolution medium [31].

2.8. In vitro cytotoxicity activity

In vitro cytotoxicity studies were performed on L929 (mouse fibroblast cell line). L929 cells were cultured in Dulbecco's modified Eagles Medium (DMEM) which was supplemented with 10% fetal bovine serum (FBS), Pen-Strep (1%, HIMEDIA) at 37 °C under 5% CO_2·The cytotoxicity of Cat-β-CD/5-FU loaded Alg/Chi **nanoflowers** were investigated in L929 cells by a 3(4,5-dimethylthiazol-2-yl)-2.5 diphenyltetrazolium bromide (MTT) cytotoxicity assay [32]. The cells were seeded at a density of $2 \times 10_4$ cells/well in 96-well cell culture plates and pre-incubated for 24 h prior to **nanoflowers** treatment. The cells were then treated with the **nanoflowers** at various concentrations of 5-Fu ranging from 20 to 100 μg/mL in medium (pH 7.4) for 24 h. Finally, the cells were incubated with 100 μl MTT containing medium (0.1 mg/mL MTT in medium) for 4 h; the medium was removed, and the formazan crystal formed in the living cells was dissolved in 100 μl DMSO per well. Relative cell viability (RCV) was calculated based on absorbance at 570 nm using a microplate reader (Infinite 200 PRO-TECAN). Viability of non-treated control cells was arbitrarily defined as 100%.

3. Results and discussion

3.1. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of native cyclodextrin and its quaternized derivative are presented in Fig. 2 a and b. In the spectrum of native CD (Fig. 2a), the broad band around 3293 cm^{-1} is due to the stretching vibration of hydroxyl groups. The absorption band at 2973 cm^{-1} can be attributed to –CH stretching vibration, and the bands at 1019 and 1152 cm^{-1} correspond to symmetric and asymmetric C—O and C—O—C stretching, respectively. As already observed in the case of cyclodextrin, band at 3307 cm^{-1} for CD derivative (Fig. 2b) is associated with the O—H stretching vibrational modes belonging to different OH groups.
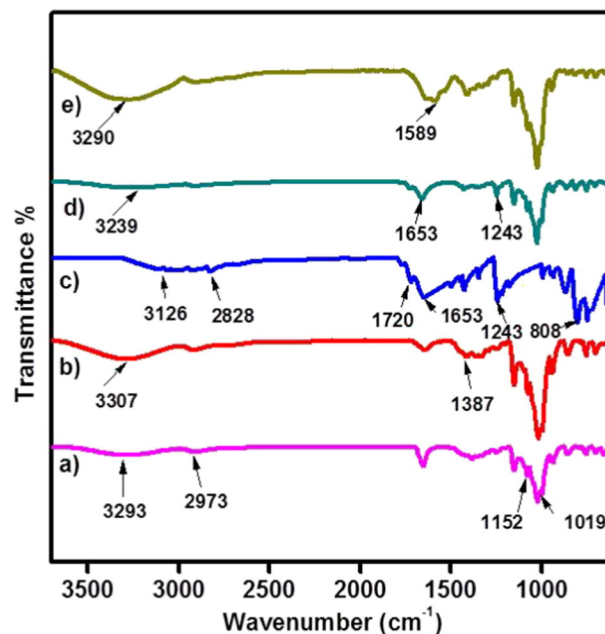

Fig. 2. FTIR spectra of a) β-CD, b) Cat-β-CD, c) 5-FU, d) Cat-β-CD/5-FU complex and e) Cat-β-CD/5-FU in Alg/Chi **nanoflowers**.

It also exhibited additional absorption band at 1387 cm^{-1} contributed by the methyl substituent of quaternary ammonium groups [33].

The FTIR spectra of pure 5-FU and their inclusion complex are shown in Fig. 2 c and d. The spectrum of 5-FU (Fig. 2c) showed several absorption bands in the range of 641–994 cm^{-1}, 1180–1720 cm^{-1} and 2828–3126 cm^{-1}. The broad band at 1653–1720 cm^{-1} was due to structural vibrations, involving overlap of C=C, C=N and C=O, while the bands at 1243 and 2828–3126 cm^{-1} correspond, respectively, to C—F and aromatic ring =C—H bonds [34,35]. The characteristic band of C—F and C=O at 1243 and 1653 cm^{-1}, respectively, were observed in the spectrum of the inclusion complex (Fig. 2d). The band at 3239 cm^{-1} is due to an OH stretching vibration with a shift in frequency. By comparing the position and intensity of these bands in the spectra, the change in the signal intensity and different shifts of wavenumbers suggests that inclusion complex was formed.

The FTIR spectrum of the Cat-β-CD/5-FU complex loaded Alg/Chi **nanoflowers** is shown in Fig. 2e. The broad and the intense peak of chitosan at 3290 cm^{-1} can be attributed to the combined peaks of O—H stretching vibrations and intermolecular hydrogen bonding along with the overlapped N—H stretching from primary amines from same region [36]. Additionally, new peak at 1589 cm^{-1} can be assigned to the formation of polyelectrolyte complex *i.e.* electrostatic interaction between carboxylic groups of alginate and ammonium groups of chitosan [37]. Characteristic band of 5-FU at 1243 cm^{-1} is observed in the inclusion complex thus confirming the presence of drug within Cat-β-CD/5-FU complex. However, when the complex was coated with chitosan in the formation of **nanoflowers**, the same bands showed a greatly reduced intensity which has been observed in other systems [38].

3.2. Nuclear magnetic resonance (^{1}HNMR and 2D NMR)

The results of ^{1}HNMR spectrum gave evidence of successful synthesis of quaternized cyclodextrin. As shown in Fig. 3a, the ^{1}HNMR spectrum of CD derivative shows a singlet at 4.82 ppm corresponding to H1 of glucose unit. Signals at 3.27–3.68 ppm were related to the H2-H6 protons of the CD. Signal at 2.50 ppm represent 2-hydroxypropyl ether segments; this result is in accordance with reported spectra [39,40], indicating successful modification of cyclodextrin. ^{1}HNMR is powerful tool which can provide supporting evidence for the inclusion
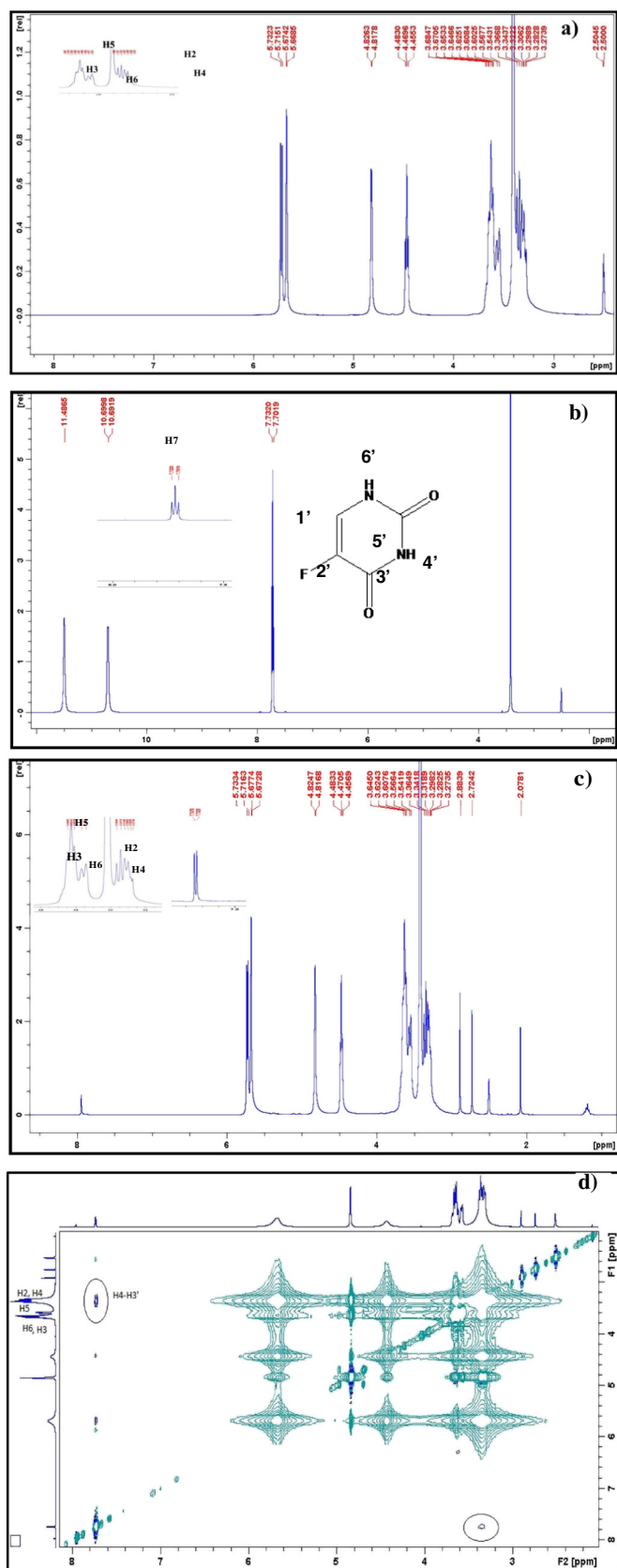
of a guest molecule into the CD cavity. The formation of inclusion complex can be confirmed from the variations of chemical shifts of host or guest in ^{1}HNMR spectra [41]. Fig. 3a, b and c exhibits the ^{1}HNMR spectra for Cat-β-CD, 5-FU and their inclusion complex. From the ^{1}HNMR spectrum of the inclusion complex, it is evident that cyclodextrin protons, namely, H1-H6 chemical shifts were slightly shifted downfield resulting from inclusion of the guest molecule.

Detailed intermolecular interaction between the host and guest were studied using 2D NMR spectra. The characteristic methylene protons (H6) and the well-separated anomeric H1 protons at glycosidic linkage are the two most convenient points of entry for the elucidation of 2D NMR spectra of the inclusion complex. In the ^{1}H NMR spectrum, the proton signals at δ4.82 ppm represents H1 of glucose units of Cat-β-CD. These protons were further confirmed due to the correlation with CH carbons at 101.3 ppm in HSQC spectrum (Fig. S1). Using these protons as reference, chemical shifts and correlations of all other protons of cyclodextrin were established, by studying sequential COSY coupling as shown in HSQC spectrum. After the assignment of protons, the corresponding carbon atoms were identified using the HSQC and HMBC (Fig. S2) spectra.

In the ^{1}H NMR spectrum, proton signals are observed at δ 7.73 ppm represents H3' of 5-FU. This alkene proton displayed HSQC correlation to carbon at 126.5 ppm ($J = 31.7$ Hz, split due to Fluorine on adjacent carbon). The carbon corresponding to this chemical shift is CH carbon in the [13]C (APT) experiment, confirms it as H3' proton of 5-FU. 5-FU carbon at 139.8 ppm ($J = 225.6$ Hz) and carbonyl carbon at 157.9 ppm ($J = 25.36$) were confirmed by HMBC correlation to H1' proton. The NOESY NMR spectra of inclusion complex also exhibited interesting proton interaction between 5-FU and Cat-β-CD. The H1' proton of 5-FU displayed NOESY interaction with H4 proton of Cat-β-CD. Since the H4 proton lies in the interior cavity of Cat-β-CD, formation of inclusion complex was thus confirmed (Fig. 3d).

3.3. Zeta size and zeta potential

The zeta potential of β-CD was around −25.33 mV and for Cat-β-CD around +7.95 mV which can be ascribed to the higher number of cationic groups in Cat-β-CD. In this study, the Cat-β-CD/5-FU complex resulted in a zeta potential of +8.07 mV. The inclusion complex obtained has an ability to protect the drug by an inclusion and electrostatic attraction as well as acting as an ideal carrier for anticancer drug delivery system (Table 1a).

The effect of different concentration of chitosan (NP1, NP2, NP3, and NP4) on the mean particle sizes were investigated (Fig. 4). The results indicate that the size of the nanoparticles diameter were dependent on the concentration of chitosan. It was noticed that with increasing concentration of chitosan (0.05 to 0.08% w/w) (increase of amino groups), the size of **nanoflowers** increased and zeta potential of the

Fig. 3. ^{1}HNMR spectra of a) Cat-β-CD, b) 5-FU c) Cat-β-CD/5-FU complex in DMSO d) NOESY spectrum of Cat-β-CD/5-FU complex in DMSO.

Table 1
Zeta potentials of a) β-CD, Cat-β-CD and Cat-β-CD/5-FU inclusion complex b) Encapsulation efficiencies of Cat-β-CD/5-FU inclusion complex and Cat-β-CD/5-FU complex loaded **nanoflowers** with increasing concentration of chitosan (% w/w) and its zeta potential.

Sample a)					Zeta potential (mV)
β-Cyclodextrin					−25.33
Cationic-β-cyclodestrin					+7.95
Inclusion complex (Cat-β-CD/5-Fu)					+8.07

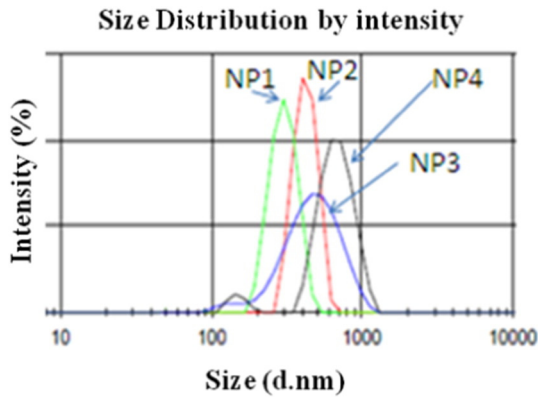| Sample b)	EE of Cat-β-CD/5-FU inclusion complex before coating (%)	Mass ratio of chitosan (% w/w)	Particle size	**Nanoflowers** (EE %)	Zeta potential (mV)
NP1		0.05	298.4 ± 1.44	76.3	+9.90
NP2	80.3	0.06	416 ± 1.09	73.7	+14.01
NP3		0.07	529 ± 0.98	78.6	+17.16
NP4		0.08	677 ± 1.12	80.4	+19.53

Fig. 4. Size distribution of several Cat-β-CD/5-FU complex loaded Alg/Chi ***nanoflowers*** measured with a dynamic light scattering spectrometer.

nanoflowers also increased as Alg/Chi ratios decreased (Table 1b). The high zeta potential can be ascribed to the higher availability of protonated amine groups with increasing chitosan concentrations. This suggests that the outer layer of the nanoparticle is mainly composed of chitosan. Increasing the concentration of chitosan could lead to increase in protection efficiency of the drug. Alg/Chi polyelectrolyte complexes are formed through ionic gelation technique when carboxylic groups of alginate interact with amino group of chitosan. The biodegradable and biocompatible polyelectrolyte complex formed protects the encapsulated material from being released more efficiently than either alginate or chitosan alone [42].

3.4. Scanning electron microscopy (SEM)

Results of SEM analysis, performed to examine the morphological aspect of pure Cat-β-CD and the freeze dried inclusion complex, are depicted in Fig. 5. Cat-β-CD appeared as irregular-shaped domains with different dimensions (Fig. 5a). However, inclusion complex appeared as a compact amorphous structure and was found to be relatively different in shape from the Cat-β-CD alone, suggesting formation of inclusion complex (Fig. 5b).

The SEM micrograph for ***nanoflowers*** is been depicted in Fig. 5c. The average particle size for ***nanoflowers*** obtained from SEM image was around 300–400 nm.

3.5. Transmission electron microscopy (TEM)

TEM micrographs of the Cat-β-CD/5-FU complex loaded ***nanoflowers*** showed distinct, dense structured spherical particles with an average size of about 270 nm (Fig. 5d). Nanoparticles when viewed in TEM appeared to be smaller as compared to average particle size observed with DLS measurements. The slightly larger value obtained *via* DLS analysis compared to TEM can be explained by dehydration of the nanoparticles during sample preparation [43].

The obtained particle size was in line with previous finding for Chitosan/Alginate nanoparticles prepared using 6:1 Chitosan/Alginate mass ratio [20]. The effect of different concentration of chitosan on the mean particle size was investigated. The ***nanoflowers*** sizes were found to be 299, 416, 529 and 677 nm for formula NP1, NP2, NP3 and NP4 respectively. The results indicate that the ***nanoflowers*** diameter depends on the concentration of chitosan as expected due to the higher
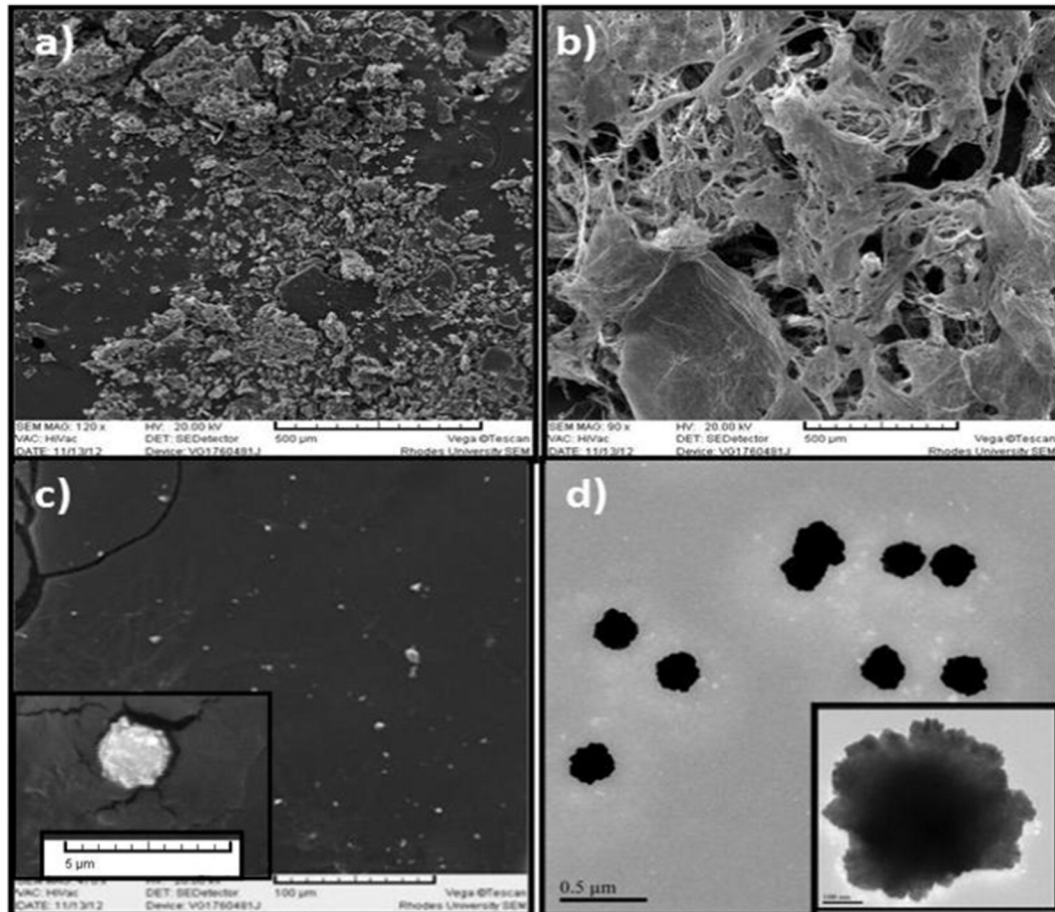

Fig. 5. SEM Micrographs of a) Cat-β-CD and b) Cat-β-CD/5-FU complex and c) Cat-β-CD/5-FU inclusion complex loaded Alg/Chi ***nanoflowers*** d) TEM micrograph of Cat-β-CD/5-FU inclusion complex loaded Alg/Chi ***nanoflowers***.

availability of positive amino groups for ionic gelation with increasing chitosan concentration. As previously reported, particle size is dependent on the concentration of the two polymers (alginate and chitosan). When the functional group of the two polymers interacts with each other in stoichiometric proportion, smaller nanoparticles are formed [44].

3.6. Differential scanning calorimetry (DSC)

DSC is a powerful analytical tool which reveals certain information about interactions and the changes that occur in the thermogram in a host guest complex. The DSC thermograms of Cat-β-CD, 5-FU and their inclusion complex are presented in Fig. 6. The DSC curve of pure Cat-β-CD (Fig. 6a) presented two broad endotherms at 100 and 315 °C; the first due to loss of unbounded water molecules and the second one attributed to degradation of cyclodextrin. The DSC curve of 5-FU (Fig. 6b) showed a sharp endothermic peak around 265 °C corresponding to its melting, followed by decomposition in agreement with reported results [34,45]. In the DSC curve of the inclusion complex (Fig. 6c), the endothermic peak around 265 °C corresponding to free 5-FU was not observed. The thermogram, however, showed loss of unbounded water molecules and apparent decomposition still present in the complex. The absence of 5-FU melting endotherm in the inclusion complex curve suggests the absence of free 5-FU in the formulation, which is consistent with inclusion complex formation.

4. Encapsulation efficiency of Cat-β-CD/5-FU complex into Alg/Chi *nanoflowers*

Encapsulation efficiency of about 80% of 5-Fu was measured within Cat-β-CD/5-FU complex. Encapsulation efficiency of 77% (EE) for Cat-β-CD/5-FU complex in Alg/Chi *nanoflowers* was measured indirectly

using UV–Vis spectroscopic analysis taking into account the difference between free portion in the supernatant and the initial amount of 5-FU used in the preparation. The high encapsulation efficiency was due to the interaction of the positively charged Cat-β-CD/5-FU complex with the negatively charged alginate polymers. The results obtained in this study indicate that percentage encapsulation of 5-FU in this Cat-β-CD/5-FU complex loaded in Alg/Chi *nanoflowers* was higher than those previously reported nanocarriers developed for delivery of 5-FU [46].

5. *In vitro* release of 5-FU from the *nanoflowers* and inclusion complex

In vitro release profiles of 5-FU from Cat-β-CD/5-FU in Alg/Chi *nanoflowers*, Cat-β-CD/5-FU inclusion complex and the pure drug (5-FU) were obtained using dialysis method (Fig. 7 a and b). The release studies were determined by incubating samples at 37 °C in phosphate buffer solutions at pH 7.4 (Fig. 7a) and pH 2.3 (Fig. 7b).

Pure 5-FU solution indicated rapid release profile with 80% of the 5-FU diffusing through the membrane within 4 h, whereas Cat-β-CD/5-FU in Alg/Chi *nanoflowers* showed sustainable release for at least 24 h. Furthermore, results showed that the rate of 5-FU release from Cat-β-CD/5-FU in Alg/Chi *nanoflowers* (pH 7.4) during first 4 h was 2× slower than that of the inclusion complex. The controlled release profile of 5-FU from the Cat-β-CD/5-FU in Alg/Chi *nanoflowers* suggests the potential of the drug delivery system for sustained release applications. According to Zhang et al. (2012) [47], a sustained release appears to be key factor for reducing hepatotoxicity of anticancer drugs and thus Cat-β-CD/5-FU *nanoflowers* could be an ideal candidate for drug delivery [47]. The release profiles at pH 7.4 and 2.3 within 24 h evidently showed that pH strongly influenced release of 5-FU from Cat-β-CD/5-FU in Alg/Chi *nanoflowers* as chitosan and alginate undergo gradual swelling, leading
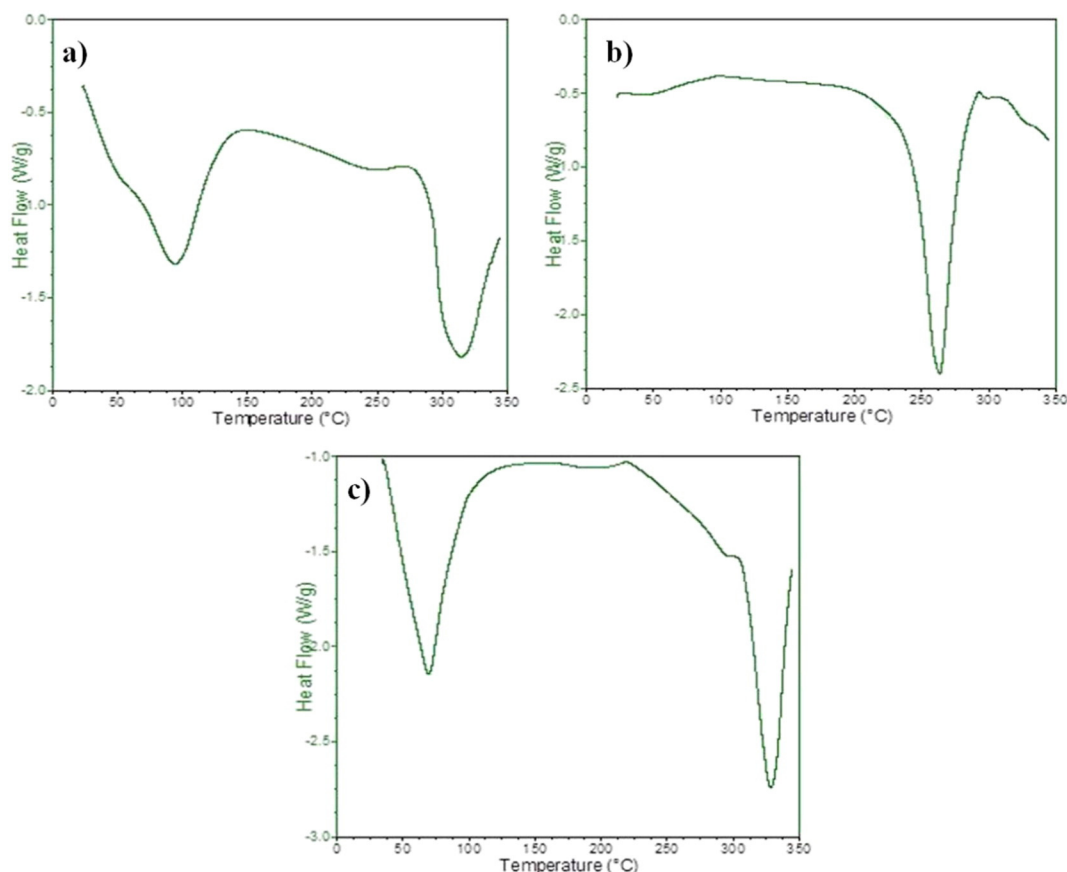

Fig. 6. DSC thermograms of a) Cat-β-CD b) 5-FU and c) Cat-β-CD/5-FU inclusion complex.
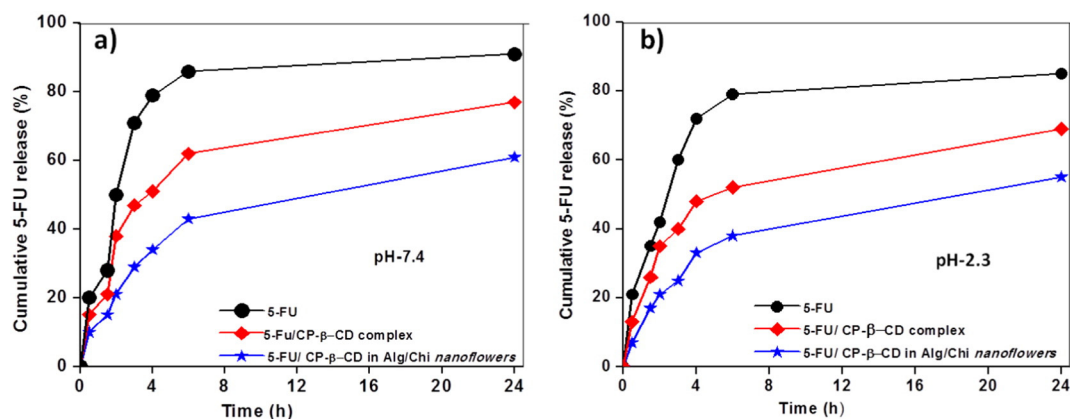
Fig. 7. *In vitro* release profiles of 5-FU, Cat-β-CD/5-FU inclusion complex and Cat-β-CD/5-FU in Alg/Chi **nanoflowers** in phosphate buffer solution a) pH 7.4 and b) pH 2.3 values at 37 °C.

to a controlled and slow release of drug from the **nanoflowers**. Notably, the release rate was high at pH 7.4 than that at pH 2.3, which was likely to be due to lesser swelling effect of the **nanoflowers** at low pH. Overall, the results indicated that if Cat-β-CD/5-FU complex or pure 5-FU is administered orally for colon specifically targeting, then about 65% drug could be lost during transportation. However, Cat-β-CD/5-FU in Alg/Chi **nanoflowers** not only inhibited releasing most of 5-FU prematurely but also assured releasing efficient quantity of the drug in the targeted area.

6. *In vitro* cytotoxicity activity

In vitro cytotoxicity activity of Cat-β-CD/5-FU loaded Alg/Chi **nanoflowers** were evaluated by the MTT assay using the L929 cell line. The range of 5-Fu (20–100 μg/mL) was maintained for Cat-β-CD/5-FU loaded Alg/Chi **nanoflowers**. As evident from Fig. 8, insignificant difference was noted in the values of the samples treated with L929 cells, in 24 h of incubation. After 24 h incubation, for different Cat-β-CD/5-FU loaded Alg/Chi **nanoflowers**, the average cell viability were 90% respectively.

7. Conclusion

A very important factor in the selection of cyclodextrin as a host for inclusion complexes is the resulting effect this has on the stability and bioavailability of the drug. This aspect, together with the sustained release characteristics of an embedded cyclodextrin complex was
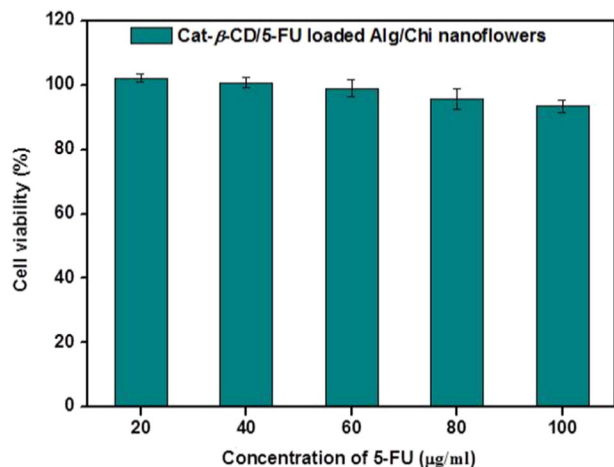
presented here. The physicochemical characterization of both the inclusion complex and the **nanoflowers** delivery system revealed that a highly efficient system could be synthesized, loaded and purified using a very simple ionotropic pre-gelation technique. Production of Alg/Chi **nanoflowers** by varying the Alg/Chi ratio demonstrated the ease with which the size of the **nanoflowers** could be controlled, for producing particles with an average size of between 300 and 700 nm selectively. Furthermore, these nanoflowers showed a very narrow polydispersity index. The encapsulation efficiency of Cat-β-CD/5-FU within Alg/Chi **nanoflowers** was found to be almost 80%, significantly higher than other reported systems, and thus the combination could serve as an ideal system for drug delivery applications. Finally, the *in vitro* release profile for inclusion complex and loaded **nanoflowers** showed an initial fast release followed by controlled and sustained release of the drug over 24 h. Additionally, the system was found to be pH dependent, non-toxic, thus indicating that **nanoflowers** were safe and can further provide a platform for tumour localized drug delivery. Thus the nanocarrier system with dual characteristic offers an interesting prospective for delivery of drug molecules and an ability to overcome problems associated with toxicity and dosing. Further *in vivo* studies are currently being undertaken to ascertain the efficacy of the systems in cell-interactions and in animal models.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.msec.2016.08.073.

Fig. 8. Cytotoxicity analysis of Cat-β-CD/5-FU loaded Alg/Chi **nanoflowers** towards L929 cells for 24 h.

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