

Pressure responsive nanogel base on Alginate- Cyclodextrin with enhanced apoptosis mechanism for colon cancer delivery

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Abstract:

5-fluorouracil (5-Fu) commonly use in the treatment of different kinds of cancer, but limited cellular uptake and death is still a problem. Herein, we report a simple process for the synthesis of pressure-sensitive nanogels that indicate to be appropriate in the delivery of 5-Fu. The hydrogels (Al-CD) prepare by crosslinking of alginate (Al) with modified beta Cyclodextrin (β -CD) as Crosslinker. Next, nanoparticles obtaine by an emulsification method. 5-Fu as model drug loades into the Al-CD nanogels easily by mixing it in aqueous solution with the nanoparticles. The results reveale that the Al-CD nanogels are cytocompatible. They also have a noticeable drug encapsulation ($82.1 \pm 5.7\%$) while they can release (in vitro controlled) 5-Fu in

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conditions that imitate the intravascular pressure conditions. These nanogels can rapidly be taken up by HT-29 cells (a colon cell line). Additionally, a higher 5-Fu intracellular accumulation and a significant cell death extension by apoptosis mechanism is notice when compare with free 5-Fu. Accordingly, the developed nanogels can be employe as an excellent candidate to overcome the inefficiency of 5-Fu in anticancer treatments and possibly can employe for further evaluation as a chemotherapical agent in applications beyond cancer.

Keywords: Cyclodextrin; Crosslinked alginate hydrogel; Nanoparticles; 5-Fluorouracil (5-FU); Apoptosis.

Introduction:

Cancer is one of the most serious causes of death worldwide. Among different kinds of cancer, colorectal cancer (CRC) was ranked as the fourth leading reason of cancer-related death for both men and women worldwide in 2008, 1.2 million new CRC cases and 0.6 million deaths(1). In the same vein, chemotherapy as one of the main common methods for the treatment of the cancer, this deadly malady, is gaining researchers' particular attention and support.

The problem lies in the fact that although chemotherapy is an effective method against cancer, most of available chemotherapeutic medicines kill both cancerous cells and normal ones resulting in serious unavoidable toxicity and side effects(2). Moreover, many anticancer drugs have serious problems with water-solubility and stability which reduce their bioavailability and clinical application. Researchers in this field have made attempts to address the above-mentioned problems regarding chemotherapeutic agents(3).

Even though anti-cancer drugs are mostly prescribed at maximum tolerated dose to eliminate as many cancerous cells as possible, the efficiency of chemotherapy in preventing CRC progression to an advanced stage is unfortunately poor. The issue lies in anticancer drugs themselves as they

cannot reach cancer cells in effective and fatal concentrations. For instance, in case of oral administration, drug release, absorption, and metabolism in the stomach and small intestine diminish drug delivery to the colon(4). In addition, the colonic mucosa seems to be a further barrier to drug permeation and to reach cancer cells located in the colon membrane wall. Based on the clinical importance of oral anticancer drugs for colon cancer, different approaches have been investigated by researchers such as use of drug delivery systems (5-8). Colon delivery systems generally work founded upon the transit time in the upper gastrointestinal (GI) tract, sensitivity to colonic pH or increased peristaltic pressure in the colon(9, 10). The peristaltic pressure offers a more reliable “trigger” for drug delivery compared with transit time or pH change. This has been investigated by a variety of polysaccharide delivery devices including chitosan, gelatin and alginate in the form of hydrogel (11-16).

Among different polysaccharides, the seaweed-derived alginate, which is a family of unbranched copolymers, is composed of alternating blocks of 1-4 linked -l-guluronic acid (G-block) and -D-mannuronic acid (M-block) residues. These have been widely investigated for the production of pharmaceutical dosage forms involving colon delivery devices. The copolymer can be cross-linked in aqueous media to produce a hydrogel at room temperature through ionic interactions with multivalent cations (e.g., Ca^{2+} , Al^{3+})(17). Alginate can prepare a compact gel at low gastric pH that hinders drug release. However, the hydrogel swells while it is slowly eroded in dissolution media by replacing the multivalent cations with physiological ions such as Na^+ and K^+ , resulting in gradual release of the loaded drug(18). Rahman et al., (19)produced alginate spheres containing 5-FU which is coated with Eudragit S100, dissolving at pH 7.0, and avoiding drug release in the higher GI tract.

5-Fluorouracil (5-FU) is one of the most commonly used chemotherapy drugs in anticancer therapy. It has become valuable in dealing with variety of human malignancies including gastrointestinal cancer, breast cancer and head and neck cancer(20). The common administration of 5-FU is intravenous (IV) injection. After IV injection, 5-FU is carried via the blood circulation system to major organs of human body, such as liver, lung, heart and kidney, resulting in wide systemic distribution and therefore decreased effective concentration of drug in target tissues with extreme side effects. However, the metabolism of drug can reduce the effective concentration of drug delivered to the colon. It has been reported that up to 85% of injected 5-fluorouracil (5-FU), is quickly converted by Dihydropyrimidine dehydrogenase to inactive agent, resulting in a half-life of 10–15 min for 5-FU (21-25). The inconvenience and worry about IV drug using via injection are further disadvantages of 5-FU administration. However, the oral drug delivery of 5-FU reduces delivered dosage of drug to the colon based on drug release, absorption, and metabolism in the stomach and small intestine(26). He et al.(27) mentioned that after oral administration of 5-FU pellets in rats, 5-FU concentrations in the stomach and intestine could reach 90 and 11 mg/g tissue, respectively. While the concentrations in the cecum and colon were <1 mg/g, either for tissue or content. According to these results, there is an urgent need to develop an ideal formulation to load 5-FU efficiently in order to overcome some of the above mentioned drawbacks. This includes the fabrication of nanoparticles (NPs) formulation that would efficiently encapsulate and sustain released 5-FU (11, 28-30). A potential restriction of common controlled drug-delivery systems is that they have been planned to function under static conditions. However, mechanically dynamic conditions are the norm of many positions in the body (e.g., compression in cartilage and bone, tension in muscle and tendon, and shear force in blood vessels). Although many researchers have worked to develop drug-delivery systems,

which actively respond to external stimuli such as temperature, pH, ultrasound, electric and magnetic fields, the pressure signals responsive hydrogels have not been systematically investigated as an external stimulation for controlled drug delivery in the human body (31-33).

In the present study, we investigate a new controlled release system based on alginate hydrogel composed of β -Cyclodextrin Crosslinker (Al-CD). Al-CD synthesis by adding the β -Cyclodextrin derivative junction point with alginate as a main chain polymer, which is sensitive to pressure stimulus. β -Cyclodextrin (β -CD) is a well-known polysaccharide which forms inclusion complexes with a variety of guests as host molecule. Therefore, β -CD has been extensively used as an excipient to improve the physicochemical and pharmaceutical properties of drug molecules. The major factors contributed to binding are supposed to be primarily van der Waals and hydrophobic interactions. Briefly, the attraction of guest molecules towards β -CD mainly depends on conformity between the guest molecules and the β -CD cavity, which is based on the terms of van der Waals interactions owing to size and shape matched between the host and the guest. Since its potential energy is contrariwise proportional to the sixth power of the distance, small alteration and/or restriction in the molecular structure of β -CD may encourage a change in its ability to interact with a guest by applying an outside stress (34). An external stress or pressure application can bring about distortion and/or conformational restriction of β -CD moieties in Al-CD as a result of the β -CD-containing network. This decreases inclusion ability of the β -CD moieties leading to the guest release.

In this study, the chemical structure of the prepared polymer was characterized by FT-IR and H-NMR spectroscopy. The synthesized polymer was then employed to prepare nanoparticles for encapsulation of 5-FU as a drug model. The TEM, FE-SEM and DLS techniques were used to

characterize the prepared particles size. In addition, drug loading via encapsulation efficiency nanoparticles was analyzed and the results were compared with 5-FU as control. In vitro investigation of 5-FU release from nanoparticles was carried out to evaluate nanoparticles with 5-FU under pressure.

2. Experimental:

2.1 Materials

Medium-molecular-weight alginate was obtained from Sigma-Aldrich (USA). β -cyclodextrin, Para toluene sulfonyl chloride (Tosyl Chloride), pyridine, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-Hydroxysuccinimide (NHS), Poly vinyl alcohol (PVA), Calcium chloride, Diethyl ether, Ethylene diamine, HPLC grade dimethylsulfoxide, sodium chloride, sodium azide, Absolute ethanol, hydrochloric acid, Aerosol OT, methanol, methylene chloride and sodium hydroxide were purchased from Sigma-Aldrich (Switzerland). 5-FU was kindly gifted from Osve Pharmaceutical Co., Ltd. (Tehran, Iran).

2.2 Preparation of Tri -O- Sulfonyl β - Cyclodextrin (CD-OTS)

CD-OTS was prepared by the method previously reported with a slight modification(35). Briefly, 2.2 gr of β -Cyclodextrin was dissolved in 22ml pyridine at 40 °C; then 1.2 gr of Tosyl Chloride was added. The reaction continued overnight. Then the reaction mixture was cooled at room temperature. The mixture purred suddenly in excess amount of diethyl ether. The precipitate was vacuum filtered and washed three times with acetone and dried in vacuum oven for 48 hr.

2.3 Preparation of Sodium Alginate - β -Cyclodextrin Hydrogel (Al-CD)

To prepare the hydrogel, first (Tris- deoxy- aminoethylamino) β -Cyclodextrin (CD-Crosslinker) was synthesized. In order to prepare the CD-Crosslinker, 3 gr CD-OTS which prepared previously, was added to 10ml of Ethylenediamine at 60 °C and stirred for 24hr (fig. 2), then

cooling to room temperature. The CD-Crosslinker was prepared by precipitation of mixture with excess amount of ethanol. The product filtrated and washed with ethanol and dried in vacuum oven over night. The hydrogel prepared by adding 0.6 gr EDC and 0.36 gr NHS to Sodium Alginate (AL) solution (1.8% w/w). The mixture was stirred for 15 min to pre activate the combination. Next, 0.68 gr CD-Crosslinker was added slowly. The mixture stirred vigorously with high speed stirrer and vacuum filtrate. Finally, the blend was allowed to dry at room temperature in Vacuum Oven for 24 hr. Alginate based hydrogels with different CD-Crosslinker content were prepared (Table I).

2.4 Synthesis of Hydrogel nanoparticles

Nanoparticles were prepared by emulsification crosslinking from the processes used in the previously mentioned procedure(36). Briefly, previously prepared polymer solution in water (1.0 % w/v; 1 ml) was emulsified into freshly prepared Aerosol OT (AOT) solution in methylene chloride (5 % w/v; 1 ml), then solution sonicated for 1 min over an ice bath and then afterward 15 ml of aqueous PVA solution (2 % w/v) was added to the above mentioned emulsion and again sonicated for at least 1 min over an ice bath to form a secondary water-in-oil-in-water emulsion. The emulsion then stirred using a magnetic stirrer, and 5 ml of aqueous calcium chloride solution (60 % w/v) was added drop wise to the emulsion. The emulsion was kept stirred with the 1000 rpm at room temperature for about 18 h to evaporate methylene chloride. To preparing drug-loaded nanoparticles, the drug (5 mg) was dissolved in the aqueous polymer solution, and the synthesis procedure continued the same as the above mentioned method. The formed nanoparticles were then collected from the solution by ultracentrifugation (SIGMA 3-16 K Centrifuge, UK) at 20,000g. Furthermore, the separated nanoparticles washed two times with

distilled water to remove excess PVA and the free drug. Finally, nanoparticles were freeze dried and stored at 4°C.

2.5 Drug loading capacity and encapsulation efficiency

Drug loading capacity (LC) (w/w %) of nanoparticles was determined according to the following formula.

$$\text{LC (\%)} = (\text{mass of 5-FU extracted from freeze-dried nanoparticles}) / (\text{total mass of freeze-dried nanoparticles}) \times 100$$

Firstly, the nanoparticles were freeze-dried and dissolved in ethanol and the 5-Fu content of the nanoparticles was determined on an Agilent Technologies HPLC system (Agilent Technologies Inc, 1200, California, USA) equipped with a UV detector (Agilent Technologies Inc, 1200) and reversed phase column (ODS C18, 5 μm , 4.6 mm \times 250 mm, Dikma, China). The mobile phase consisted of 70-30 Acetonitrile-water (v/v %), respectively. The mobile phase was pumped at a flow rate of 1.0 ml/min and 5-FU concentration was measured at 279 nm.

Encapsulation efficiency (EE) of nanoparticles was calculated according to the following formula:

$$\text{EE \%} = (\text{Total amount of 5-FU inside Nanoparticles}) / (\text{Total amount of 5-FU in collected nanoparticles})$$

2.6 In vitro drug release

In vitro release of encapsulated 5-FU from the nanoparticles was measured under sink conditions (volume of release medium used was enough to dissolve at least five times the quantity of the drug present in nanoparticles). Briefly, 100 mg of nanoparticles was dispersed in 10ml of phosphate-buffer saline (PBS, pH 7.4) and placed into a dialysis bag with molecular weight cutoff of 12000 D (Sigma, Germany). The dialysis bag was placed in a flask containing 100 ml

of release medium (PBS), pH 7.4) (sink condition). The whole assembly was shaken at 100 rpm and 37°C. At the predetermined time intervals, 5 ml of the release medium was removed and replaced with the fresh medium. The content of 5-FU in the medium was measured by HPLC. The cumulative release percentage of 5-FU was calculated and compared with the CLE used as control.

The Kinetic of drug release from the nanoparticles was calculated by investigating its related data according to Korsmeyer-Peppas model:

$$M_t / M_\infty = Kt^n$$

where t corresponds to the release time (min), M_t / M_∞ is a fraction of drug released at time t , k is a kinetic constant related to the network structure and n is a diffusion constant which indicates the mechanism of release. As the similar diffusion constants obtained in all the release assays, no other mathematical models were applied.

2.7 Release of 5-FU responsive to pressure

In order to measure the effect of pressure on the release of nanoparticles, release was performed in sink condition which was described in 2.6, but putting pressure on the samples was an exception. Briefly, 100 mg of previously prepared nanoparticles was dispersed in 100 ml PBS, and then placed in cylindrical glass. The Glass was then covered with the PTFE door and sealed. The Cylindrical glass was equipped with three tubes, one for applying pressure (by exploiting the Nitrogen with computer control pump), one for extracting the samples and inserting the freshly PBS (4 way junction), and finally one for naming the pressure gage demonstrating the pressure. Pressure-resistant cell (cylindrical glass), immersed in a water bath to regulate and stabilize temperature (measured accuracy within 0.05°C) and placed on the stirred bar. The whole assembly was stirred at 100 rpm and 37°C. The pressure inside the cell was generated and

released through a pump and was measured with a pressure gauge (measured accuracy within 2 mmHg). A pump operated by computer was used to adjust and apply the cyclic pressure (fig. 1). At the predetermined time intervals, 5 ml of the release medium was removed and replaced with the fresh medium. The pressure was kept constant throughout the experiment by applying Nitrogen through by the computer controlled pump. The content of 5-FU in the medium was measured by HPLC as described previously. The cumulative release percentage of 5-FU was calculated and compared with the no pressured samples.

2.8 Cytotoxicity study

MTT assay was performed HT-29 cell line (National cell Bank of Iran, Pasteur Institute, Tehran, Iran) in order to evaluate the cell cytotoxicity and viability of the Al-CD nanoparticles. Cells were seeded at a density of 1×10^4 cells/well onto 96-well plates (Nunc, Denmark) in Roswell Park Memorial Institute medium (RPMI) containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin/amphotericin) for 24 hours in the incubator (in humidified atmosphere with 5% CO₂ at 37 °C). Cytotoxicity of the Al-CD nanogels was determined by standard MTT assay with an indirect extract process (ISO 10993-12). Briefly, Al-CD nanogels were treated in an autoclave (15 min at a pressure of 15 ponds and temperature of 121°C), then removed in extraction medium (a RPMI with 10% FBS) for five days. The extract was gathered, and added to the full-growth media of confluent HT-29 cells to achieve three concentrations of 10, 25 and 50 $\mu\text{g}\cdot\text{ml}^{-1}$ and incubated for 24 hr, then 100 μL PBS containing of 0.5 $\text{mg}\cdot\text{ml}^{-1}$ MTT was added to each cell, and cells were once more incubated for 4 hr at 37 °C in CO₂. Then the resulted formazan crystals were dissolved by solubilisation buffer. The optical density of the solution was measured at a wavelength of 545 nm.

2.9 Induction of apoptosis

Apoptosis (programmed cell death) represents a major causative factor for the anticancer action of a drug (37, 38). Therefore, in the present study, flow cytometry was used to investigate whether apoptosis can bring about the cell death induced by drug loaded crosslinked alginate nanogels.

Human colon cancer cell line HT-29 was pretreated with 5-FU loaded nanogels, pure Al-CD-3 and free 5-FU for 24 hr, and was analyzed for various parameters of apoptosis. Annexin-V-FLUOS staining assay was used to determine the plasma membrane alterations in cells. In order to prepare the cells for test, the cells were washed in PBS, resuspended in 100mL of binding buffer containing a FITC conjugated anti-annexin V antibody. They were then analyzed with a flow cytometer (FACS Calibur, Becton Dickinson, and Bedford, MA). A standard sample, 10,000 cells per treatment condition, was analyzed by flow cytometry using an excitation wavelength at 488 nm and emission 610 nm.

3. Result and discussion

3.1 Characterization of synthesis polymer

The synthesis of Al-CD consists of three steps, first, activation of the hydroxyl groups of β -CD by reacting with tosyl chloride. Second, reaction of CD-OTS with ethylenediamine to prepare CD-Crosslinker. Thereafter, the carboxyl groups of Sodium alginate react with amino groups (NH_2) of CD-Crosslinker to prepare the Al-CD (Fig.2). Different weight ratio of Sodium Alginate and β -Cyclodextrin were employed in order to optimize the hydrogel characteristics (Table I).

Figure.3 represented the FT-IR spectra of CD, Tosylated β -CD (CD-OTS), CD-Crosslinker, Sodium alginate (Al) and prepared Al-CD. Infrared (IR) spectra were recorded on a Shimadzu 8400S Spectrometer (Shimadzu 8400S FT-IR, Osaka, Japan) with a scanning range from 4000 to

400 cm^{-1} . The FT-IR spectra of β -CD indicate the broad band at 3384 cm^{-1} which is assigned to the symmetric and asymmetric OH stretch. The peaks at 1157, 1080, and 1028 cm^{-1} refer to C-O stretching vibration of glucosidic bonds, C-O stretching vibration in cyclic alcohols, and C-O stretching vibration in primary alcohols, respectively (39). The assigned peaks for CD-OTS are as the following: stretching vibrations at 1637 and 1488 cm^{-1} corresponded to aromatic rings and the peaks at 1335 and 1155 cm^{-1} that are assigned to the symmetric and asymmetric SO_2 stretching. The FT-IR spectra of CD-Crosslinker show a broad peak at 3300-3500 cm^{-1} which can be assigned to O-H and N-H groups, the peak at 1560 cm^{-1} refers to the bending vibration of N-H groups and the 1370 cm^{-1} corresponds to C-N stretching vibration. The FT-IR spectra of sodium alginate (Al) indicated a broad peak at 3422 cm^{-1} and the peaks at 1615 and 1416 cm^{-1} which correspond to acidic and alcoholic O-H groups and asymmetric and symmetric stretching vibration of carboxylate ion salt, respectively. The FT-IR spectrum of final hydrogel shows the characteristic bands of amide for hydrogel at 1644 cm^{-1} for amide I and 1566 cm^{-1} for Amide II band that coupled with the C=O stretching vibration.

^1H NMR analysis confirms the proposal hydrogel formation:

^1H NMR CD-Crosslinker: (300 MHz, D_2O , DSS) δ = 2.57-2.66 (m, 12H, $-\text{NHCH}_2\text{CH}_2\text{NH}_2$), 2.70-3.01 (m, 6H, $-\text{CH}_2(\text{H}-6')$ -), 3.22-3.33 (m, 3H, $-\text{CH}(\text{H}-4')$ -), 3.40-3.48 (m, 14H, $-\text{CH}(\text{H}-2, \text{H}-2')$ -), $-\text{CH}(\text{H}-5')$ -), $-\text{CH}(\text{H}-4)$ -), 3.72-3.80 (m, 19H, $-\text{CH}(\text{H}-3, \text{H}-3')$ -), $-\text{CH}(\text{H}-5)$ -), $-\text{CH}_2(\text{H}-6)$ -), 4.92 (bs, 7H, $-\text{CH}(\text{H}-1, \text{H}-1')$ -) ppm.

^1H NMR Al-CD: (300 MHz, D_2O , DSS) δ = 2.7-2.8 (6H, CD- NCH_2 -), 2.8-3.01 (12H, $-\text{CH}_2$ -AL, $-\text{CH}_2(\text{CD}, \text{H}-6')$ -), 3.03-4.1 (Sugar protons (AL, CD), DOH), 4.9 -5.2 (CD, AL(anomeric protons), 8.01 ($-\text{NHCO}$ -AL) ppm.

3.2 Differential Scanning Calorimetric (DSC)

Thermal analysis by DSC of polymers in different parts of synthesis procedures was undertaken using a Mettler-Toledo DSC821. Measurements were performed over the temperature range of 0–400 °C at a heating rate of 10°C/min. On accounts of simple and interpretable spectra compared to many other characterization methods, the Differential Scanning Calorimetric (DSC) method has been largely developed for characterization of polymer materials. As it is detected in the DSC spectrum of the native alginate, the endothermic peak at 106 °C is ascribed to the water absorption related to the hydrophilic nature of the functional groups of native alginate(36). On the other hand, in the DSC spectrum of the CD, an endothermic peak at about 118 °C was observed due to dehydration process and an exothermic peak at about 246 °C was detected due to the oxidation of OH groups of sample. The endothermic peak shifted to the 115 °C and exothermic peak converted to endothermic at 206 °C respectively for CD-Crosslinker, These indicate that the number of OH groups was reduced. However, it exposed the substitution reaction with amino groups of ethylenediamine which took place on the OH group of CD. In addition, the exothermic peak at 281 °C was also observed as a consequence of the oxidation of amino group. DSC thermograms of final hydrogel indicate an endothermic peak at about 110 °C which is broader than alginate because of dehydration of water molecules that bind to CD molecules and alginate. They also represent two exothermic peaks at about 270 and 290 °C , which can refer to the oxidation of carboxyl group at AL and amino group at CD-Crosslinker, respectively. Figure. 4 DSC spectra of: A) AL, B) CD, C) CD-Crosslinker, and D) AL-CD (Hydrogel)

3.3 Particle size and morphology of nanoparticles

The dispersion and aggregation of nanoparticles are important for their application; hence, measuring of the particles size is always a mandatory procedure. Furthermore, particle size can

affect biological characteristics of nanoparticles and the sub-hundred nanometer particle size is supportive in the drug delivery systems, since nanoparticles in this size range have been shown to have higher cellular and tissue uptake (40, 41). The nanoparticle size in solution was analyzed by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS. The particles size distribution spectrum for the Al-CD nanoparticles is represented in Figure. 5 that illustrate the average particle size of 55.1 ± 5.1 nm and polydispersity index (PDI) of 0.213.

The FE.SEM image which illustrated the morphology and size of nanogels is represented in Figure. 6. The Figure. 6 shows that most of nanoparticles are in spherical shape with an average diameter < 50 nm. TEM image in Figure. 7 indicate nanoparticles shapes are in agreement with the FE.SEM image in spherical morphology. The difference in average particle size measured by DLS might be observed because of the swelling of nanoparticles in the solution during the measurement.

3.4 Drug loading and encapsulation efficiency

Drug loading and encapsulation efficiency of the Al-CD is represented in (Table I) which demonstrates dependency on the Al-CD concentration. Increasing the concentration of CD from 0.1 to 0.85 in the formulation of nanoparticles led to an increase in 5-FU encapsulation from 40 to 88%. We found a non-linear relation between the amount of drug added to the formulation and encapsulation efficiency. The enhanced loading capacity of the nanogels for 5-FU was driven from CD-Crosslinkers that could operate as binding sites to involve and complex 5-FU within the CD-Crosslinkers cavities. This could be explained according to the contribution of hydrophobic-hydrophobic interaction between CD and the Drug. Raising the concentration of Al-CD, it was expected to increase the number of CD groups available for interaction with 5-FU and subsequently better drug entrapment in the nanoparticles. Nevertheless, in sample 5 high

crosslinking density decreased the loading efficiency of sample because the flexibility of nanoparticles was reduced flexibility of nanoparticles by increasing the crosslinker which resulted in a lower loading capacity for this sample (42, 43).

In order to calculate drug loading capacity of nanogels, we used different concentrations of nanogel solution (Al-CD-4) and drug (5-Fu) (Table II).

These data show that 1ml of Al-CD-4 solution (10000ppm) loaded 88.1% of 5-Fu (5000ppm) and this is the highest capacity of nanogels for loading 5-Fu because the increase in nanogel or 5-Fu concentration does not show any significant change.

These nanogels are made of Alginate and Cyclodextrin that both could load 5-Fu. Infact, there are two molecules which could load drug by encapsulation, entrapment, and inclusion complex.

Other studies proved that modified polymers based on alginate have a high drug loading capacity, that is more than 70% for 5-Fu (44-46).

3.5 In vitro drug release

The application of Al-CD nanoparticles to deliver 5-FU was monitored by drug release rate.

Release rate was calculated by the following formula:

Cumulative 5-FU release (%) = Cumulative amount of 5-FU released at time t / initial amount of 5-FU $\times 100$

Figure. 8 show that Al-CD-2 released 5-FU faster than Al-CD-3 in consequence of the lowest CD content and crosslinking degree. The same data procedure was investigated for Al-CD-4 for which the drug release was the slowest among three samples. An initial burst release of loaded 5-FU was observed within the early hours that can be attributed to free drug molecule that attached to the surface of samples. In addition, no burst release was observed for samples and controls

during the initial period of investigation. Apparently, inclusion complexation of 5-FU with CD delayed the diffusion of 5-FU from the hydrogels. After the early burst release, the release rate reduced greatly for a certain amount of time. At this stage, the release rate was enhanced again until all loaded 5-FU was discharged. Particularly, the two samples AI-CD-3 and AI-CD-4 followed the distinctive multiphasic release profile more. During the period of reduced release, it is understood that the 5-FU molecules were retarded by the durable binding of CD Crosslinkers. After the 200 hr, the release was enhanced because of the degradation of the nanogels by the medium of disbanding of the nanogel together with the drug.

The release mechanism of 5-Fu drug was analyzed with the Korsmeyer-Peppas equation:

$$M_t / M_\infty = Kt^n$$

Where M_t/M_∞ is the fractional amount of the 5-FU released at time t ; n is the diffusion exponent indicating the release mechanism; and K is a characteristic constant of the system. From the slope and intercept of the plot of $\log (M_t/M_\infty)$ vs. $\log t$, kinetic parameters including n and K were calculated.

(Table II) indicated that R^2 is higher than 0.90 and also n is lower than 0.5 in all samples. It can be perceived that the 5-FU release kinetics was based on the Fickian diffusion. This Fickian behavior may offer that the 5-FU release from the nanoparticles is controlled by the drug diffusion process. Based on our results, we find that the majority of observed release is going to be due to diffusion of the entrapped drug molecules through the polymer membrane, and not due to the drug absorption in the polymer. We consider that after cross-linking the polymer captured drug solution and let the drug diffuses over time. Eventually, we think that because of degradation, more drug molecules in solution will have a chance to release. Thus we consider the drug release controlled by diffusion and degradation of polymer.

The study of drug release kinetics using the zero-order and first order kinetic models were carried out. The respective correlation coefficients (R) were determined by means of the linearization of dissolution profiles. It is found that the first-order kinetic model is more appropriate for nanoparticles (the one that presented a higher value of correlation coefficient) (Table III).

The release of the drug which followed first order kinetics can be expressed by the following equation:

$$dC/dt = -Kc$$

$$\text{Log } C = \log C_0 - Kt/2.303$$

Where K is the first order rate constant expressed in t^{-1} , C_0 is the initial concentration of drug, and t is the time. The data obtained are plotted as log cumulative percentage of drug released vs. time which would yield a straight line with a slope of $-K/2.303$ [51].

3.6 Release of 5-FU responsive to pressure

Investigation for the releasing rate under the accelerated pressure compared to non-pressure release revealed that obvious changes in drug release rate can be attributed to binding and non-binding of drug molecule by β -CD (Fig.9). These results illustrated that the connection mode of the β -CD residues within the nanogel structure plays an important role in drug release. It is very important to mention that the regulation of the host-guest interactions controlled the drug release rate in response to the pressure. The drug release from Al-CD can be increased by applying the pressure since it can change the inclusion ability of β -CD moieties. Generally, based on Le Chatelier's principle, molecules assembled with increased pressure reduces their total volume when the release is faster in comparison to non-pressure condition (30). Our investigation clearly indicates that the release rate of drug (5-FU) was monitored by different parameter such as

crosslinking density, β -CD content and active site of β -CD. However the binding ability of β -CD moieties in Al-CD can be one of important parameter in controlled release of drug from sample since applying pressure can change the release rate of sample. The pressure sensitivity of samples can be attributed to the β -CD groups that contained a molecular network. This network can interact with drug and release the drug by applying the pressure. Based on the ability of prepared sample for controlling release with stimulating factors, prepared sample can be used for promoting drug delivery.

3.7 Cytotoxicity assay

Cytotoxicity of Al-CD-3 on HT-29 cells was studied using MTT assay (fig. 10). As can be seen in Fig. 10 high viability (95-98% as compared to control) of HT-29 cells is significant when exposed to 10, 25 and 50 $\mu\text{g ml}^{-1}$ of Al-CD-3. These results indicate that Al-CD-3 in this concentration range displays a nontoxic effect on HT-29 cells and they are biocompatible.

3.8 Induction of apoptosis

As shown in Figure. 11, cells exposed to Al-CD nanoparticles without drug have significant apoptosis since alginate does not exert any influence on cancer cells. However, a significant increase of apoptotic cells (66.3%- 43.36%) was observed in the cancer cell lines, upon exposure to loaded Al-CD nanoparticles samples 3 and 4, respectively in comparison with the free 5-Fu (33.51%) as control. The apoptosis mechanism was employed approximately two times more in this experiment because cellular uptake of 5-fu from nanoparticles was higher than the same concentrations of free 5-FU.

It is clear that there were a higher percentage of cells showing apoptosis exposure to sample-3 compared to the sample-4. This can be explained by the increased release of 5-FU within cancer

cells that can be attributed to the faster release of the drug from the nanoparticles acting on their target drug release section.

Although nanoparticles have a tendency to accumulate in cancer cells via passive targeting development, this passive strategy has restrictions because of its nonspecific delivery approach. However, when nanoparticles have polysaccharides parts, polysaccharide moieties can cooperate definitely with the biological targets, e.g., lectins that have been found in the surface of malignant cells and cancerous tissues (47). Therefore, nanomaterial with polysaccharides structure could increase cellular uptake and cancer-targeting abilities (48).

In addition, based on Yang, et al, the increase in apoptosis can be traced to polysaccharides based nanoparticles (49). (The apoptotic data were listed in table IV).

4. Conclusion

In this study, we developed a formulation for preparation of Alginate-Crosslinked by modified β -Cyclodextrin to improve the biocompatibility and to make it sensitive to pressure. Drug loaded nanoparticles were synthesized successfully and its potential to control delivery of 5-FU was investigated. The obtained nanoparticles were characterized via NMR, FT-IR, DSC, FE-SEM, DLS, and TEM. The 5-FU loaded nanoparticles showed a controlled drug released property. The complexation of 5-FU with β -CD crosslinkers largely increased the loading level of 5-FU in the nanoparticles. The complexation could also reduce the initial burst release effect. Later on, it can retard the releasing of the complex 5-FU for a certain period until the hydrogels started to degrade hydrolytically and all remained 5-FU was released. The data demonstrated that β -CD in the alginate-based hydrogels played a dual role as Crosslinker as well as a binding site for hydrophobic anti-cancer drug, which imparted promising drug loading and controlled release behavior to the hydrogels.

The nanogels displayed a high cytocompatibility and could effectively enhance apoptosis in HT-29 cells. In summary, the usage of improved nanogels as carrier for 5-Fu delivery leads to a developed in vitro anticancer efficiency which could make them as promising nanoparticles for the proficient delivery of anticancer drugs. Moreover, the formed AI-CD nanogels presenting pressure-controlled drug release sensitivity could be applied as a suitable delivery system in brain cancer or adrenal tumors which experience raised intracranial pressure or raised ICP (50) and high blood pressure (51).

Figure captions

Fig. 1 Schematic of proposed device used for measuring the drug release from nanoparticles under pressure.

Fig. 2 The process of AI-CD preparation.

Fig. 3 Fourier transforms infrared spectra.

Fig. 4 DSC spectra of: A) AI, B) CD, C) CD-Crosslinker, and D) AI-CD (Hydrogel).

Fig. 5 The effective diameter and size distribution of encapsulated nanoparticles measured by particle size analyzer.

Fig. 6 FE-SEM images of AI-CD nanoparticles.

Fig. 7 TEM image of AI-CD nanoparticles that negative stained with 2% w/v uranyl acetate.

Fig. 8 In vitro release of 5-FU from encapsulated nanoparticles for different sample preparation (Means \pm S.D, n=4).

Fig. 9 comparison of in vitro release of 5-FU from AL-CD-3 sample without and under 180, 220 and 240 mmHg pressure (Means \pm S.D, n=4).

Fig. 10 MTT assay: effect of (10, 25 and 50 $\mu\text{g ml}^{-1}$) of Al-CD-3 on the viability of HT-29 cell line for 24 hr.

Fig. 11 apoptosis assay by FACS, **A)** HT-29 cells as control, **B)** Exposed cell to 10 $\mu\text{g/ml}$ of free 5-FU, **C)** Exposed cell to 10 $\mu\text{g/ml}$ of Al-CD-3 without drug loaded, **D)** Exposed cell to Al-CD-3 with 10 $\mu\text{g/ml}$ of 5-FU drug loaded, **E)** Exposed cell to Al-CD-4 with 10 $\mu\text{g/ml}$ of 5-FU drug loaded.

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Table I. Different ratio of CD-Crosslinker and alginate used of synthesis Hydrogel and Drug loading and encapsulation efficiency of Al-CD nanoparticles (means \pm S.D, n=5).

Sample	Alginate(gr)	CD-Crosslinker(gr)	EDC(gr)	NHS(gr)	Loading of drug %
1	1.14	0.33	0.29	0.17	60.44 \pm 0.9
2	0.1	0.51	0.45	0.27	81.98 \pm 1.6
3	0.82	0.68	0.6	0.36	80.41 \pm 1.8
4	0.65	0.85	0.75	0.45	88.1 \pm 2.1
5	0.51	0.1	0.87	0.53	40.56 \pm 1.4

Table II. Calculation of drug loading and encapsulation efficiency of different concentration of Al-CD-4 nanoparticles and 5-Fu.

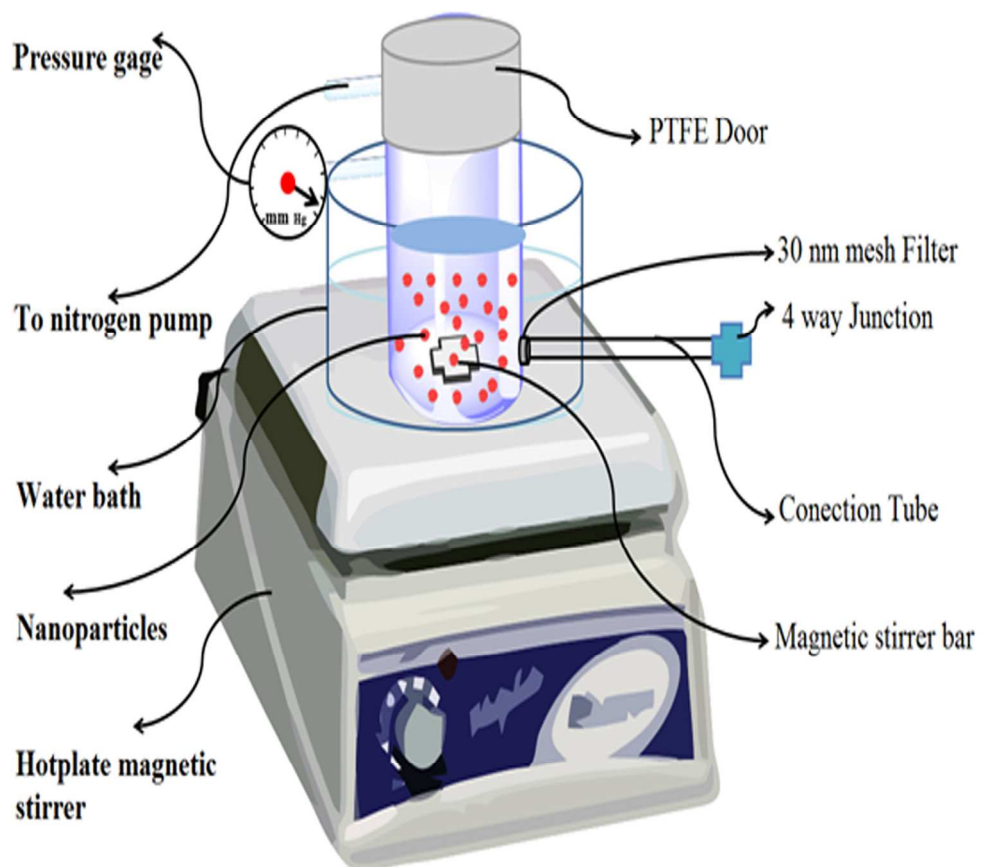
Volume of nanogels solution with 10000ppm concentration(ml)	Drug concentration(ppm)	Drug Loading (%)	Encapsulation efficiency (%)
0.5	5000	40.48 \pm 0.8	80.96 \pm 1.5
0.75	5000	33.22 \pm 0.9	83.06 \pm 1.02
1	5000	29.37 \pm 0.9	88.10 \pm 1.5
2	5000	20.76 \pm 1.6	83.06 \pm 2.01
1	1250	8.19 \pm 0.7	73.72 \pm 1.08
1	2500	15.88 \pm 1.26	79.44 \pm 1.6
1	7000	35.82 \pm 2.17	87.31 \pm 2.5

Table III. Calculation of kinetics and diffusion constant for 5-FU release from AL-CD nanoparticles using Korsmeyer-Peppas equation and zero order release.

sample	Reaction condition	Korsmeyer-Peppas model			Zero order model	
		n	K	R ²	K	R ²
AL- CD2	Simulated biologic Environment	0.481	0.272	0.90	0.132	0.92
AL- CD3		0.481	0.251	0.91	0.131	0.93
AL- CD4		0.439	0.228	0.90	0.129	0.90

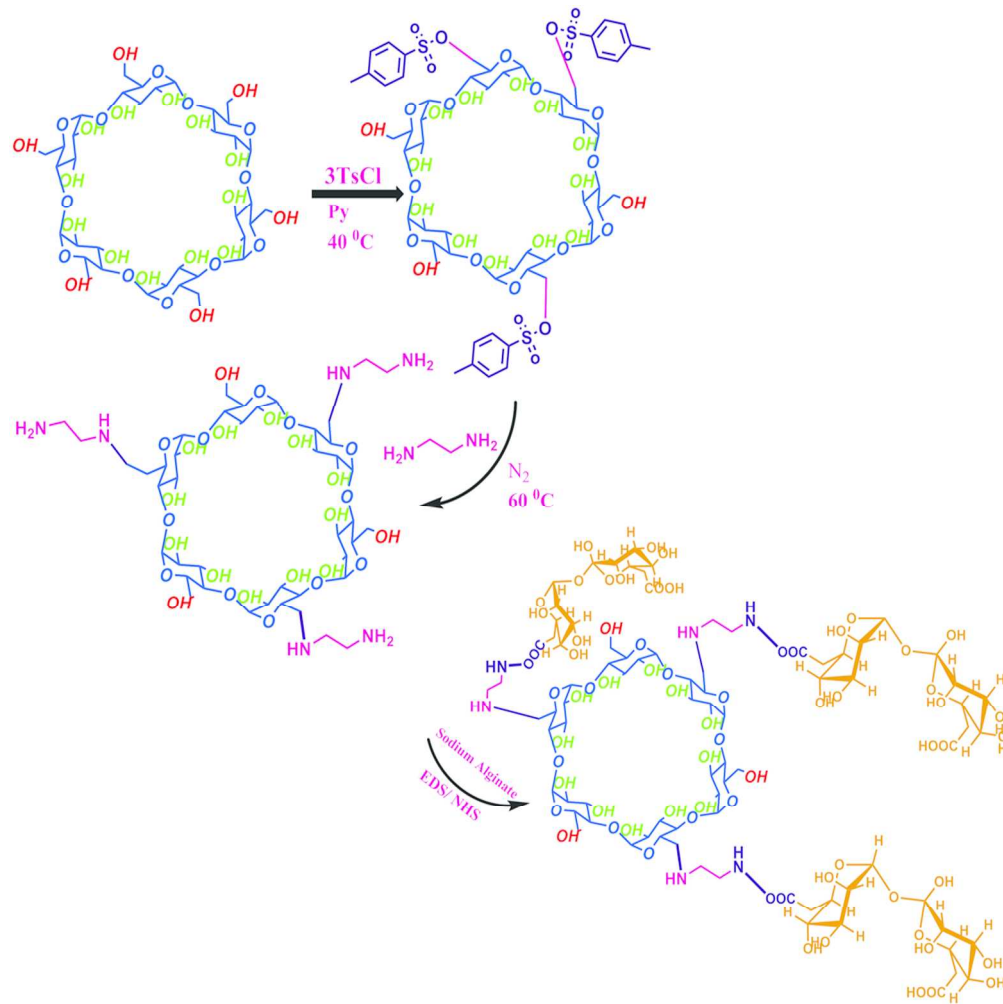
Table IV. Apoptotic data extracted from flowcytometry analysis (means \pm S.D, n=5).

Sample	Apoptosis (%)	Necrosis (%)	Secondary mechanism (%)	Living cells (%)
HT-29 cells as control	0.41	0.2	3.74	85.84
AI-CD-3 without drug	10.11	2.78	2.84	84.18
AI-CD-3 loaded 5-FU	66.30	3.84	14.08	20.98
AI-CD-4 loaded 5-FU	43.36	9.84	15.89	30.91
Free 5-FU	33.51	9.77	5.50	51.21



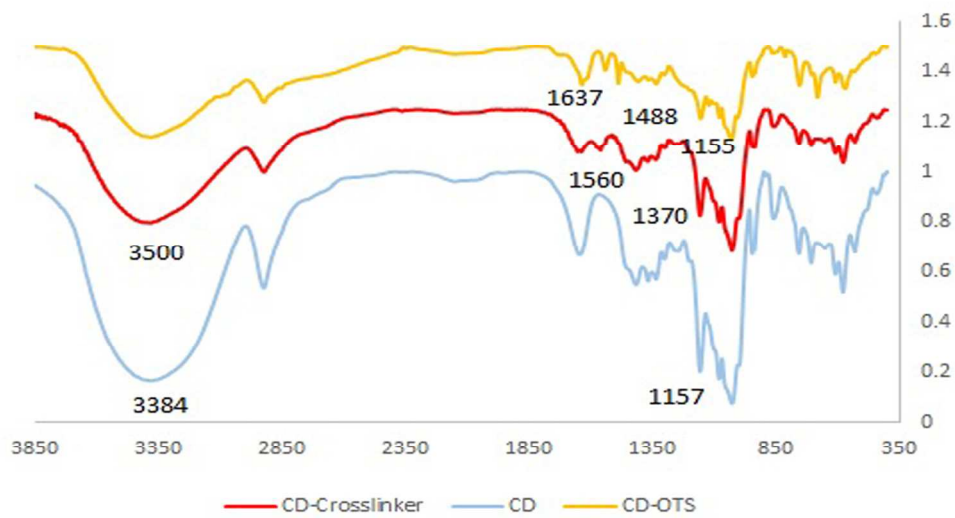
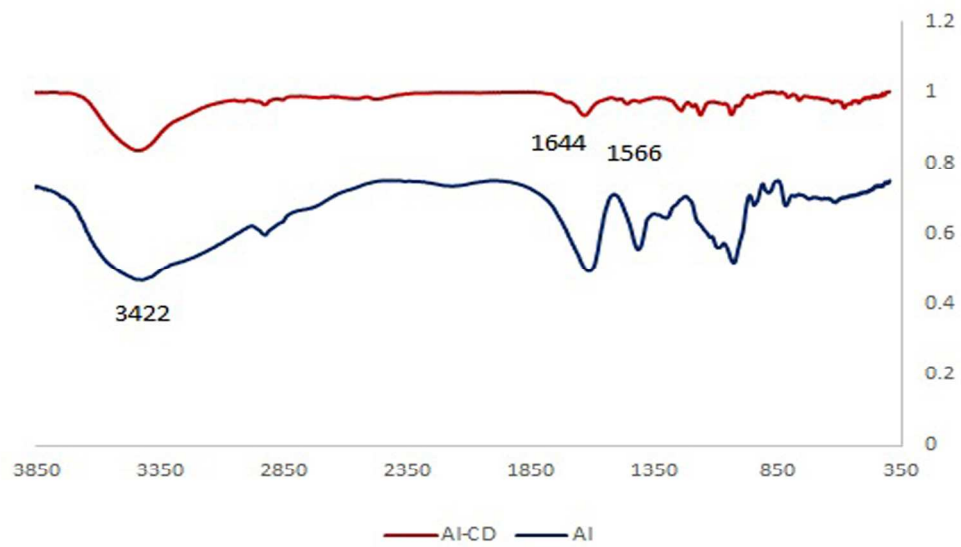
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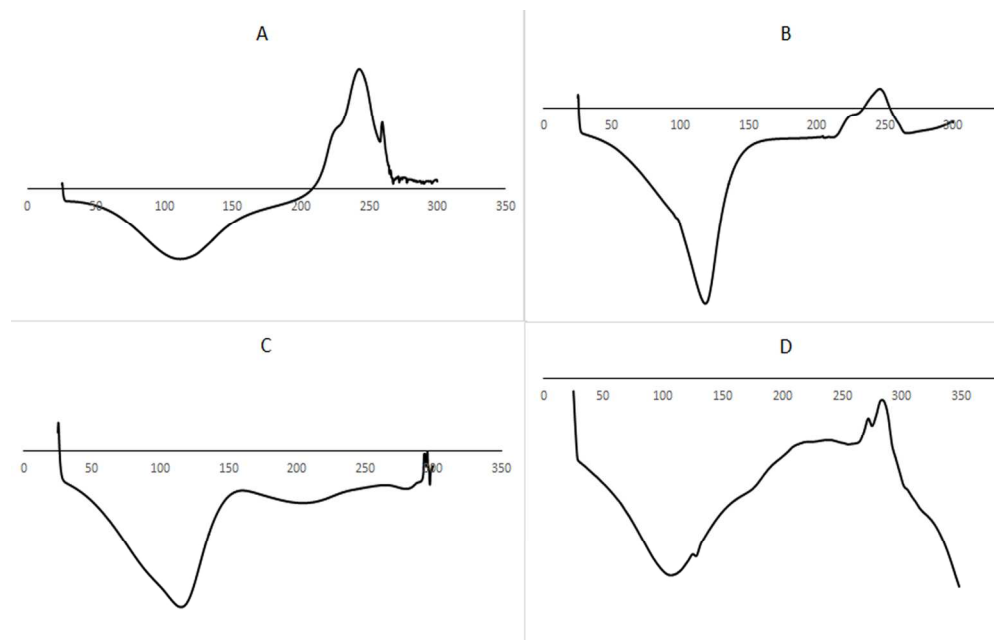


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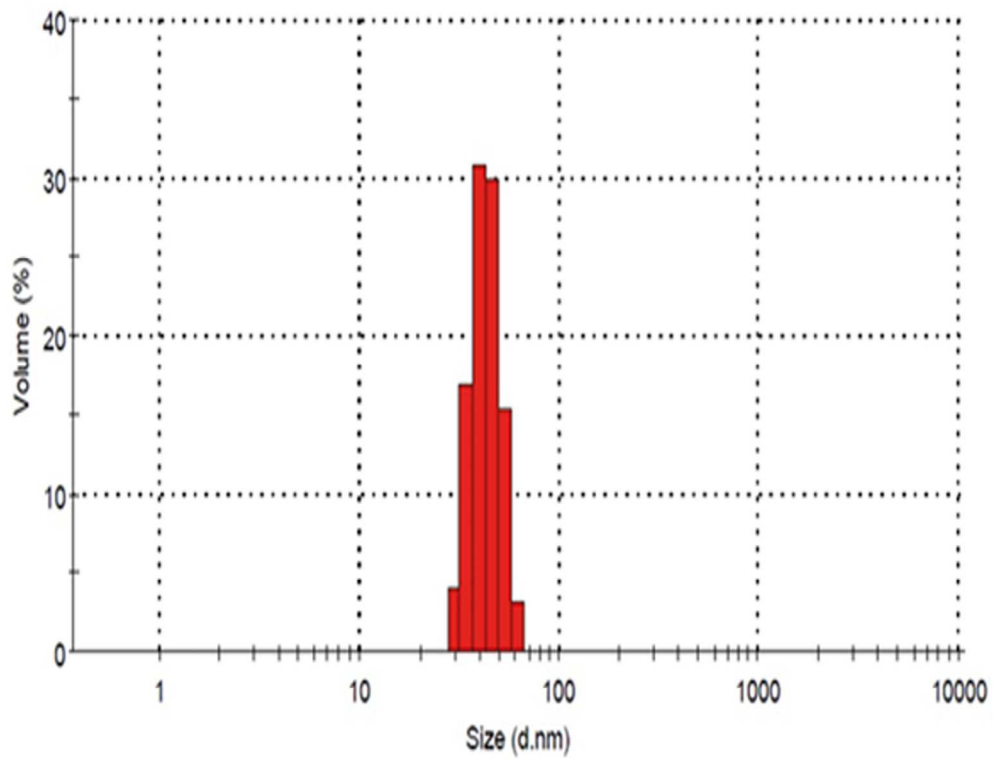


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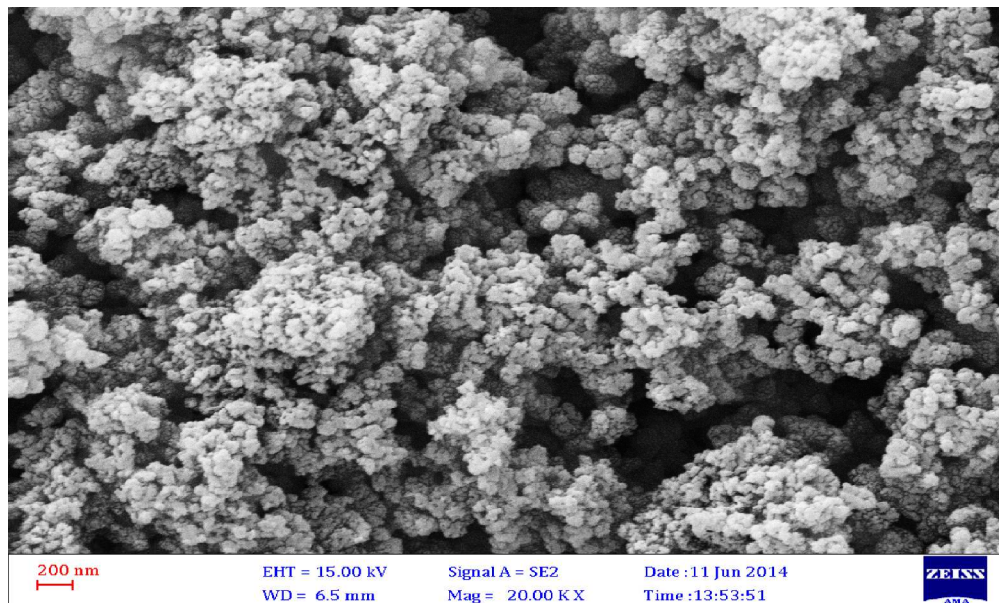
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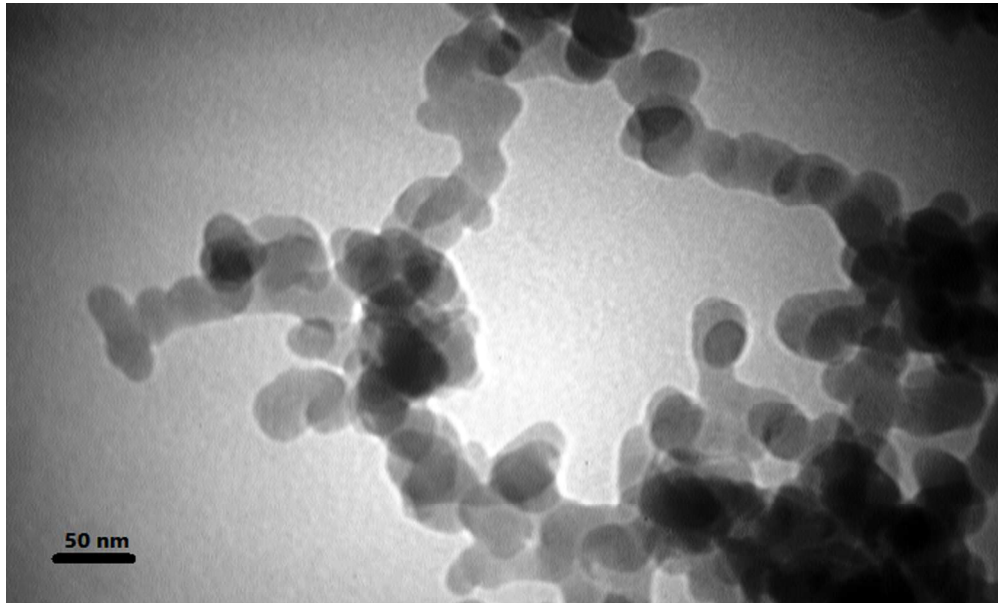
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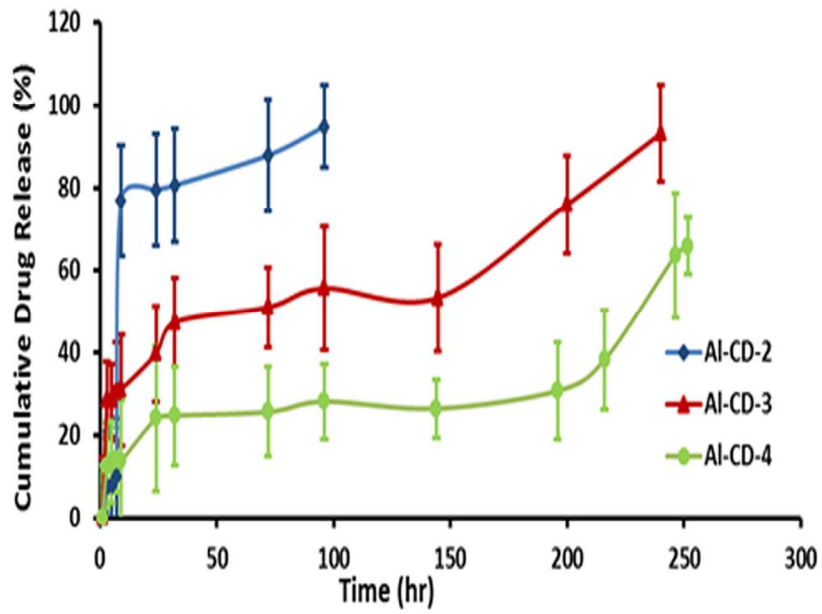
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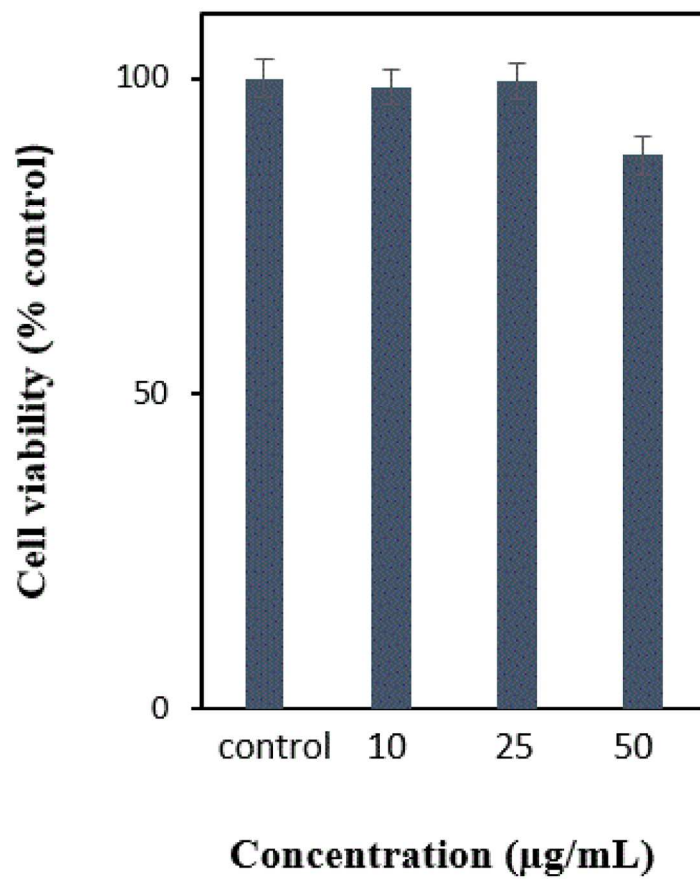
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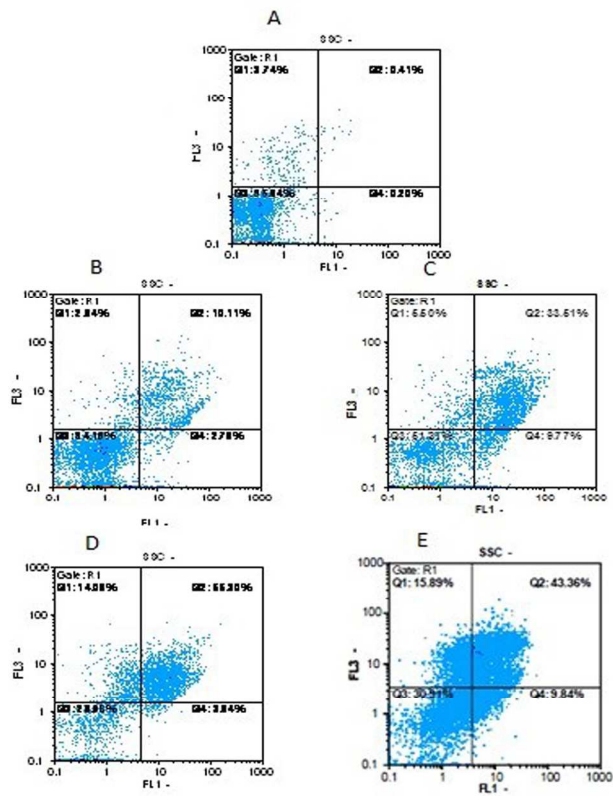
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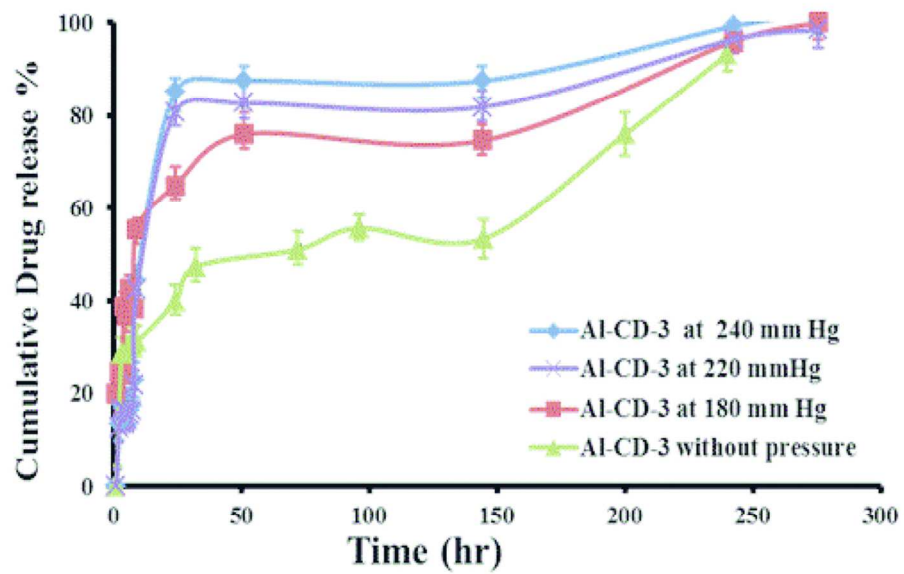
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