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Thermosensitive polymers-based injectable hydrogels: a quantitative validations design utilized for controlled delivery of gefitinib anticancer drug

A.A.H. ABDELLATIF[1,2], A. AL-SUBAIYEL[1], A.M. MOHAMMED[2]

[1]Department of Pharmaceutics, College of Pharmacy, Qassim University, Qassim, Saudi Arabia
[2]Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy, Al-Azhar University, Assiut, Egypt

Abstract. – OBJECTIVE: Gefitinib (GFB) was loaded into different designs of thermos- and pH-responsive polymer-based hydrogels, namely chitosan (CH) and Pluronic F127 (Pl F127) with the aid of a crosslinking β-glycerophosphate (β-GP).

MATERIALS AND METHODS: GFB was loaded in CH and P1 F127 hydrogel. The preparation was characterized and tested for their stability and efficacy as antitumor injectable therapy devices. The antiproliferative effect of the selected CH/β-GP hydrogel formula was investigated against the hepatic cancerous cell, HepG2 using the MTT tetrazolium salt colorimetric assay. Furthermore, the pharmacokinetic was performed for GEF using a developed, reported and validated LC method.

RESULTS: All hydrogel samples showed no changes in color, separation(s), and crystallization in both liquid and gel forms. The CH/β-GP system showed a lower viscosity (110.3 ± 5.2 C_p) compared to CH/β-GP/Pl F127 system (148.4 ± 4.4 C_p) in the sol phase. Also, the results confirmed a continued increase in rats' plasma during the first four days (T_{max}) with a plasma peak level (C_{max}) of 3.663 µg/mL followed by a decrease below the detection limit after 15 days. Moreover, the results indicated no significant difference ($p < 0.05$) between the predicted and observed GEF-concentration data and that the proposed CH-based hydrogel facilitated its sustained release as distinguished from the longer value of MRT of 9 days and an AUC0-t of 41.917 µg/L/day.

CONCLUSIONS: The medicated CH/β-GP hydrogel formula had a higher targeting-controlled efficiency against a solid tumor than the free poor water soluble GFB.

Key Words:
Gefitinib (GEF), Chitosan (CH), Pluronic F127 (Pl F127), β-glycerophosphate (β-GP), Antiproliferative effect, Thermos- and pH-responsive polymers-based hydrogels.

Introduction

Gefitinib (GEF) is a selective transmembrane glycoprotein epidermal growth factor receptor (EGFR) specific tyrosine kinase (TK) receptor inhibitor[1]. EGFRs can initiate signals which consider important regulatory factors in the proliferation[2,3]. There is a correlation between EGFR overexpression and increased malignancy or poor prognosis in many human cancers[4]. EGFR is a major factor in several solid tumors[5,6]. GEF, an anilinoquinazoline (N-(3-chloro-4-fluorophenyl)-7-methoxy-6-(3-morpholin-4-ylpropoxy)quinazolin-4-amine) has a low molecular weight of 446.9 Da, effectively used in several solid tumors overexpressed EGFR[7-9]. GEF is a water-insoluble dibasic compound with two different acidic dissociation constants (pK_a of 5.28 and 7.17), giving its pH-dependent solubility in gastrointestinal fluids after an oral administration. Unfortunately, its bio-absorption is limited as its limited dissolution, consequently, its bioavailability is low[6]. The oral bioavailability after a single dose of GEF (Iressa® tablet, 250 mg, AstraZeneca) was 57%. Moreover, an increase in the gastric pH of more than 5 reduced its bioavailability to 47%[10].

Several *in vitro* approaches[11] have been developed to improve the solubility issue of GEF tablets, which are expected to decrease the unwanted large dose and avoid the accompanying side effects such as vomiting and diarrhea. Phillip et al[12] enhanced the solubility and dissolution of GEF through an inclusion complexation with hydroxypropyl βCD (HP-β-CD) and randomly methylated βCD. They concluded that adding hydrophilic polymers, namely polyvinyl pyrrolidone or hydroxypropyl methylcellulose to

Corresponding Authors: Ahmed A.H. Abdellatif, MD; e-mail: a.abdellatif@qu.edu.sa
Ahmed M. Mohammed, MD; e-mail: ahmedabdelaal@azhar.edu.eg

HP-β-CD significantly improved the dissolution profile of GEF. Next, an *in vivo* study[1] was developed after the enhancement of the solubility of GEF, depending on the spray drying (SD) solid dispersions technique. It was reported[13] that the GEF-SD group had a 9.14-fold increase in the area under curve (AUC) compared to free drugs with the same oral dose (50 mg/kg). When GEF is administered in an oral dosage form will accumulate in the tumor side, skin, liver, and kidney as well, at a concentration significantly higher than those required in the plasma because of its hydrophobicity[13-15]. Through the present project, we hypothesized that another proper drug delivery such as the parenteral hydrogel approach could be circulated to utilize the efficacy of GEF as a selective EGFR inhibitor.

Many scholars[15] have been done on hydrogels and their practical biomedical applications. These applications include biocompatibility, non-toxicity, biodegradability, sol-gel transitions, adjustable characteristics, and prolonged or controlled release of medicinal compounds. It could be advised to deliver GFF systemically, avoiding the 1st pass metabolism[16]. Specifically, a thermosensitive hydrogel is considered a sol-togels sensitive to temperature. The therapeutically released kinetics of a GFE-incorporated smart hydrogel may be regulated by ambient conditions or external stimuli such as redox potential, low pH, illness status, or specific enzymes[17]. Furthermore, chitosan polymer-based hydrogel has great promise for usage as an injectable *in situ* inert and shows negligible inflammatory responses[16,18-20]. Therefore, the scope of the current project is developing a thermosensitive chitosan-based hydrogel for the administration of GEF into cancer cells line, namely HepG2 cells, under regulated conditions. Moreover, the laboratory physicochemical parameters, *in vitro* release, cytotoxicity, and *in vivo* pharmacokinetic behavior of the drug-loaded chitosan hydrogels were all assessed to determine the efficacy of the final *in situ* system. We aimed to prepare, characterize, and test the stability and effectiveness as anticancer injectable treatment devices using simple and precise procedures. Using the MTT tetrazolium salt colorimetric test, the antiproliferative activity of the CH/-GP hydrogel formula against HepG2 was determined. Pharmacokinetic investigations confirmed the continuing rise in rats' plasma over the first 4 days (T_{max}) with plasma peak level (C_{max}) of 3.663 µg/mL followed by a fall below the detection limit after 15 days.

Materials and Methods

Materials

Gefitinib (GEF) was purchased from Biosynth Carbosynth (Carbosynth Ltd, Compton, Berkshire, UK). Medical grades Chitosan (CH), β-glycerophosphate (β-GP), and Pluronic F127 (Pl F127) polymers were purchased from Fluka BioChemika (Buchs, Switzerland). Roswell Park Memorial Institute-1640 (RPMI-1640) mammalian cells' growth medium with (penicillin, and streptomycin) was purchased from (Sigma Aldrich, Co, Spruce Street Saint Louis, MO, USA). Hepatic cancer cell lines (HepG2) were obtained from Serum and Vaccine Authority in Egypt. Analytical grades acetic acid, dimethylsulphoxide (DMSO), and potassium dihydrogen phosphate (hydroxyapatite) were purchased from El-Nasr Pharm. Chem. Co., (Cairo, Egypt). HepG2 cells were obtained from Vacsera Egy, (El-Dokky, Egypt). All the other chemicals and solvents were of the highest quality.

Preparation of the Medicated Chitosan-Based Hydrogel

CH/β-GP hydrogel was prepared according to a previous method developed by Ruel-Gariép et al[21], with minor modifications. To get the final concertation of CH, 1.8 grams were dissolved in 100 mL acetic acid (0.1 M). A medical-grade CH powder was sprinkled over 0.1 M acetic acid. At the same time, it was stirred until complete dissolution. An aqueous solution of β-GP (35%, w/v) was prepared, and the solution of CH was cooled for 15 min in an ice bath. Then the cooled β-GP solution was added dropwise to the cooled CH solution in a ratio of 1:1, with a continuous stirring for 15 min. Next, the final medicated hydrogel was prepared by pouring β-GP (35%, w/v) and CH solution (1.8%, w/v in acetic acid, 0.1 M) directly into a solution of Pl F127 (10%, w/v). Then, GEF powder 1 g was added to the medicated hydrogel under continuous stirring for an hour. The resulted product was stored at 8°C for further examination.

Characterization of the Prepared Medicated CHβ-GP/ Pl F127-GEF Hydrogel

Organoleptic properties study

In both liquid and gel phases, the produced hydrogel was visually checked for certain parameters, namely clarity, fluidity, homogeneity, phase separation, and overall acceptability[22,23]. The data were scored for each parameter, and the results were manipulated as means ± standard deviation.

Rheological study

The viscosity of the medicated hydrogel was measured using an Ostwald U-tube capillary viscometer (Sigma Aldrich, Cairo, Egypt). The viscometer was filled with the studied liquid until the desired level was reached. The viscometer is placed vertically in a thermostatic bath to achieve the desired temperature. It is sucked or blasted into an opposite arm until the liquid is slightly over the mark. In this step, the suction is released, and the liquid's descent is timed[24]. Then, the viscosity of gel forms of the CH-based hydrogel system was measured with the aid of Brookfield DV-III ultra-viscometer, RV model (Brookfield Co., New York, NY, USA) using T-bar spindle (T-D 94) at 50 rpm at 37 °C. All measurements were carried out at an ambient temperature of 25 ± 3°C, and the viscosity values were displayed as centipoises. All values were given as means ± standard deviation.

pH determination study

The prepared medicated hydrogel's pH values were measured using the Ama Digital pH meter (Ama Co., Amberg, Bayern Germany). The probe of the pH meter was immersed in both the hydrogel samples, sol and gel forms, and different samples (n = 3). The checked value should be in the range of normal human skin pH of 4.5 - 7.0 to avoid any irritation after application.

Syringeability and injectability study

Syringeability (easiness of removal from vial to a syringe) and injectability (performance of the formulation during injection from pre-filled syringes) are two critical characteristics in the management of any parenteral dosage form. The syringeability and injectability were determined using a 5-mL sterile syringe fitted with a needle of (intradermal) ID 0.84 mm. Different 5.0 mL of hydrogel samples (n = 3) were filled into the syringe needle and injected into a piece of chicken meat at room temperature using finger pressure[25,26].

Gelation temperature (T_{gel}) study

In the study of the temperature at which the hydrogel system changed from sol into the gel, a standard rheological technique was used[27,28]. The 10 mL transparent vials containing different cold sample solutions (n = 3) and a magnetic bar were left in a low-temperature thermostat water bath. The temperature of the solution was elevated at a constant rate of continuous stirring (200 rpm) using a magnetic bar. The temperature at which the magnetic bar stopped its movement was measured with the aid of an immersed thermometer, and it was defined as the gelation temperature (T_{gel}).

Solubility studies

The solubility of GEF was checked practically in a fixed volume of distilled water, acetic acid (0.1 M), and phosphate buffer solution (0.1 mM, pH 7.4), carried out at 37.5°C to determine the optimum sink conditions for the drug release. In brief, an excess of GEF (20 mg/1mL) was added to the phosphate buffer media with shaking for 48 h. After an established equilibrium period, samples were taken, filtered through a 0.45 µm membrane filter, and spectrophotometrically assessed at λ_{max} 253 nm[29,30].

In Vitro Drug Release

Different GFB-loaded hydrogel sample solutions, 0.5 mL (in phosphate buffer, pH 7.4), an equivalent to 2.5 mg GEF, were placed in 2 mL Eppendorf tubes and incubated at 37°C for 1 h. Then, 1.0 mL acetic acid (0.1 M) was added, and the total mixture was stirred at 50 rpm. At several time intervals (1, 2, 4, 8, 10, 20, 40, 60, 90, and 120 min), 0.5 mL sample solution was taken and eventually replaced by a new release buffer medium. The amounts of the released GEF have been assayed for their concentration by UV spectrophotometry. All measurements were carried out in triplicate, and the results were presented as means standard deviations (means SD)[31].

Dynamic Light Scattering (DLS) Measurements

The average size distribution of the produced medicated hydrogel, as well as the released free drug, were determined using photon correlation spectroscopy (PCS) at an ambient temperature of 25°C, using a Zetasizer ZSNano (Malvern Instruments, Malvern, UK). Each run proceeded in triplicate every 100 sec. And the interval between runs was 60 sec, with a 180-second equilibrating period. To avoid auto-attenuation, thus fluctuating values, the attenuator was set at level 9. The average of three series of measurements was obtained, and the average of each measurement was utilized to compute the outcome and each measurement[32,33].

SEM Analysis

Scanning electron microscopy (SEM) was used for qualitative and quantitative estimates of the shape and arrangement of the hydrogel particles. Drops from randomly selected hydrogel

sample solutions were added on a slipcover (Sputter coater, JOEL JFC-1300). After carefully drying at room temperature, covered with a thin layer of platinum in a vacuum for 55 sec at 25 mÅ using a coating unit to make it electrically conductive before imaging in SEM instrument (JSM-6400, Akishima, Tokyo, Japan). A 10-100 K magnification microscope power and an accelerating voltage of 100 kV were used to display the sample's morphology[32,34].

Kinetics Treatment of In Vitro Drug Release Data

To describe the overall release behavior of GEF from the prepared hydrogel, zero-order = ($Q_t = Q_0 - K_0 t$), 1st-order = ($\log Q_t = \log Q_0 - \frac{K_1 t}{2.303}$), Higuchi diffusion ($Q_t = K_h \sqrt{t}$), and Korsmeyer-Peppas ($\log Q_t = \log K + n \log t$) models were used[35-37]. In the mentioned models, Q_t means that the quantity of GEF released from the hydrogel at a given time (t), with a constant release rate of K_h, denotes the diffusional exponent, which describes the kind of dissolving mechanism that was used. The correlation coefficients (r) for the parameters involved, as well as the values of diffusional exponents (n), as recommended by Burnham and Anderson[38], were used to select the best acceptable model.

In Vitro Antitumor Activity (Cell Viability Test)

The *in vitro* antitumor activities of GEF (positive control), CH-based hydrogel (negative control), CHβ-GP, and CHβ-GP/Pl F127-GEF hydrogels systems were evaluated against the hepatic cancer cell lines (HepG2) using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium) method. HepG2 cells were seeded in RPMI 1640 growth medium with a combination of penicillin and streptomycin antibiotics (1%, w/v). Cells were maintained at 37°C in a humidified incubator containing 5% CO_2[39]. 1.0×10^5 cells per milliliter of medium were adjusted and planted into a 96-well plate with 100 microliters in each well. Then, the culture medium was replaced with 0.5 mL of different dialyzed concentrations of the sample solutions of the medicated hydrogel (6.25, 12.50, 25.0, 50.0, and 100.0 µg/mL) and incubated at the gelling temperature (T_{gel}) of 37°C. After incubation, 25 µL of 5 mg/mL MTT reagent in phosphate buffer solution (PBS) was added to the cells for 4 h. Around 200 µL of DMSO was added to dissolve the formazan crystals[40]. After 5 min of shaking, the optical densities were determined at 560 nm using an ELISA microplate reader (ELISA microplate reader, Bio-Tek, Winooski, VT, USA). All experiments were performed in triplicate (n = 3), and the results were expressed as mean ± SD. The dialyzed hydrogel blank solution (negative control), and GEF blank control (positive control, at the same experimental concentrations), were treated similarly to remove the experimental biases.

Pharmacokinetic Studies

Albino rats (weighing 185 ± 15.1 g of body weight) were acclimatized at room temperature 25±3°C with a 12-h light/12-h dark cycle 24. 20 rats were divided into two independent groups; group 1 was the controlled group, administered only a blank hydrogel. Group 2 received the medicated hydrogel (in a dose of 25 mg/kg) with a syringe device (Doowon Meditec Corp., Youngin, Korea). The predetermined intervals for getting the blood samples were 1, 3, 6, 10, and 15 days, then collected and centrifuged (5,000 rpm at 10°C/5 min) to prepare the plasma for high performance liquid chromatography (HPLC) analysis. The chromatographic conditions of analysis were done on LC (Agilent technologies 1,200 series, G1321A FLD system), using reversed-phase zorbax eclipse XDB-C_{18} column (250 mm × 3 mm, 5 µm) and isocratic elution consisting of acetonitrile: phosphate buffer (0.1 mM, pH 3.6), at ration 45:55%, v/v, and a flow rate of 1.0 mL/min. The pharmacokinetic parameters were performed using PKsolver software program (WinNonlin, New York, NY, USA)[41]. The concentrations of GEF in different rats' plasma samples (n = 10) were calculated from the corresponding calibration curve equation in the developed and validated LC method. The plasma concentration-time curve (C_p/t, µg/mL × min) was constructed. Next, from the constructed (C_p/t) curve, all common pharmacokinetic characteristics of both medications were calculated using Equation 1.

$$C_p = \frac{(K_a \times F \times D)}{\{V_d \times (K_a - K_{el})\} \times (Exp - K_{el} \times t - Exp - K_a \times t)}$$

Where K_a: absorption rate constants; F: absorption fraction; V: volume of distribution; $t_l/2k_{10}$: is the terminal half-life. C_p: plasma concentration; k_{el}: the elimination rate constant. The area under $C_{p/t}$ curve (AUC_{0-t} and $AUC_{0-\infty}$) (ng × h/mL) was assessed using the trapezoidal rule.

Statistical Analysis

Unless otherwise stated, all findings were reported as mean, standard deviation (mean ± SD), and a probability value of $p < 0.05$ was considered statistically significant. Statistical analysis consisted in an analysis of covariance (ANOVA) followed by post-hoc tests.

Results

In the present study, CH-based hydrogel incorporated GEF, an anticancer agent, was successfully prepared after mixing CH polymer solutions with crosslinking agents (Pl F127 and β-GP) in an ice bath for 15 min (Figure 1).

A stirring method at 5,000 rpm at a cooling temperature for 30 minutes was used to prepare GEF-incorporated hydrogel. The qualitative description of the medicated hydrogel was done by visual examination to provide the acceptance criteria for the product's final appearance. All hydrogel samples showed no changes in color, separation(s), and crystallization in both liquid and gel forms. More specifically, the color was white and turbid in the case of the gel form but cleared colorless in the other liquid form. The prepared hydrogels' viscosity was determined in sol and gel phases. Two hydrogel systems were designed which are CH/β-GP and CH/β-GP/PI F127. Generally, the results showed a significant difference ($p < 0.05$) between them using a sample size of n = 3. The experimental trials showed that the CH/β-GP system showed a lower viscosity (110.3 ± 5.2 C_p) compared to CH/β-GP/Pl F127 system (148.4 ± 4.4 C_p) in the sol phase. On the other hand, the viscosity of the gel phase was 114 ± 8 $C_p \times 10^3$ and 120.8 ± 1.3 $C_p \times 10^3$ for CH/β-GP and CH/β-GP/Pl F127 systems, respectively.

Solubility Studies

Practically, GEF is water-insoluble and phosphate buffer (0.1 M, pH 7.4) at a temperature of 37.5°C. An acetic acid solution (0.1 M) could solubilize GEF and provide more than 10-fold sink conditions for the *in vitro* release study. As stated in the Henderson-Hasselbalch equation, a chemical ionization depends on media pH and drug pKa, thereby explaining GFB's increased solubilities.

In Vitro Drug Release

The release study of GFB from its own medicated CH-based hydrogel systems (CH/β-GP and CH/β-GP/Pl F127) was carried out in acetic acid (0.1 M, pH 2.4) at 37.5°C under the previously determined sink conditions. The results showed a significant difference in GEF release between the two prepared hydrogel systems. The complete GEF release was obtained after 7 days in CH/β-GP compared to a higher sustained release effect until 12 days in the CH/β-GP/Pl F127 system (Figure 2).

Scanning Electron Microscopy (SEM)

Figure 3A-B displayed the SEM images of the two medicated CH-based hydrogel systems. The polymeric platforms generally had a well-organized morphology, although they were diverse and difficult to describe. The lamellar layout of the CH/β-GP hydrogel formulation was observed at a higher resolution of 15 Kv allowing the observation of a more clearly defined structure, as compared to the other formulation (CH/β-GP/Pl F127).

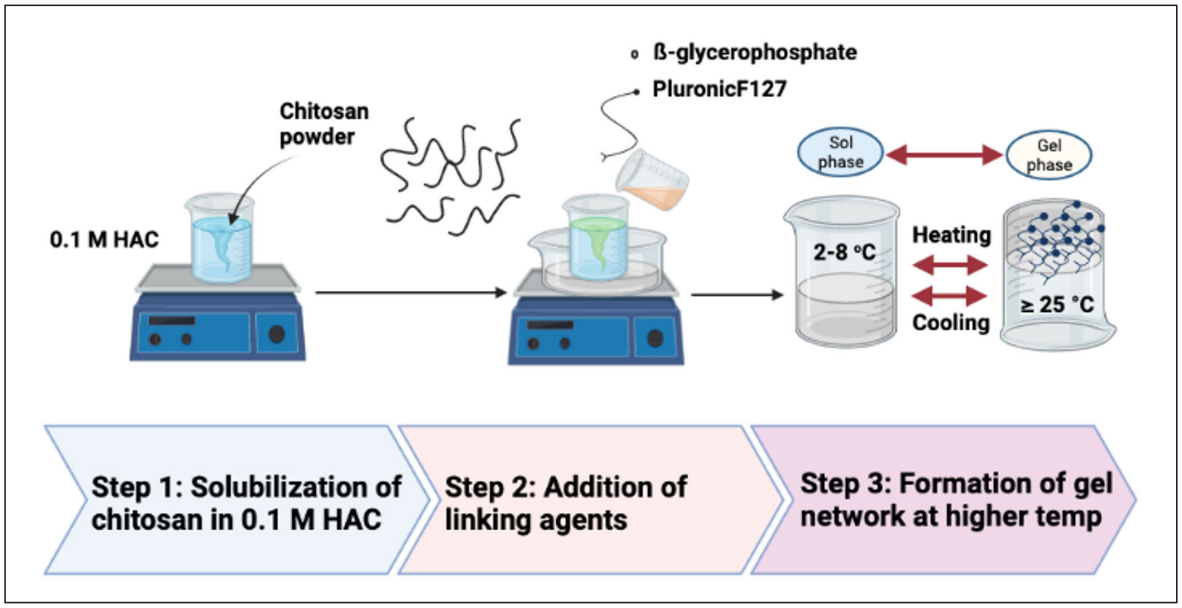

Figure 1. Graphical representation of thermosensitive CH-based hydrogel.

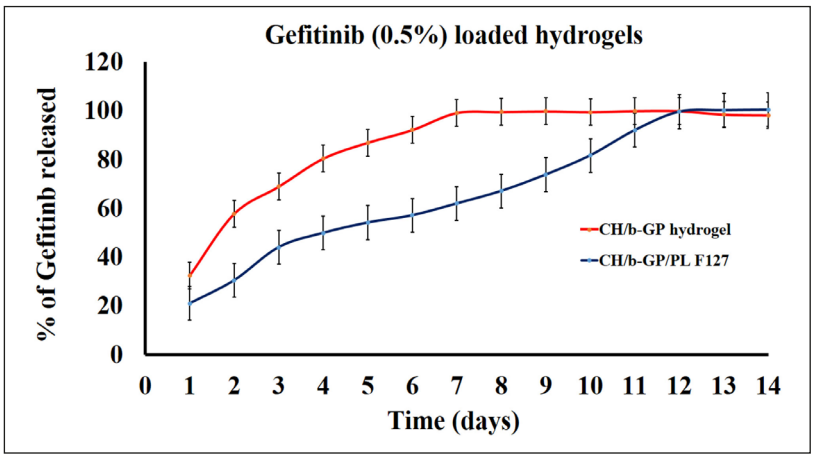

Figure 2. *In vitro* release profile of GEF CH-based hydrogel systems; CH/β-GP hydrogel (red color) and CH/β-GP/Pl F127 hydrogel (blue color).

Dynamic Light Scattering (DLS) Measurements

DLS measurements of the release media revealed a size distribution of 445 nm. The releasing media contained nano-assemblies of a biodegradable hydrogel network (Figure 4).

Kinetics Treatments of In Vitro Release Data

The highest r indicated the matched reproducible linearity of the released data and represented the actual mode of the release. To calculate the release exponent (n) for the Korsmeyer-Peppas diffusion model, we used the slope and r for each system, as illustrated in Table I and Figure 5. The result of the dissolution trial experiments (n = 3) demonstrated that the released GEF from the proposed hydrogels was characterized by fitting the Higuchi diffusion model, with correlation coefficient r values of 0.975 and 0.977, respectively, for CH/β-GP and CH/β-GP/Pl F127 hydrogel systems. In the Higuchi diffusion model, the drug release follows a square root of time relationship. The positive linearity correlation indicates that GEF is released primarily by diffusion from a porous and tortuosity hydrophilic polymetric matrix, as shown in Figure 5. The dependence of GEF release from the hydrogel's matrices suggested that the GEF release is diffusion controlled and that matrix erosion after swelling is negligible.

In Vitro Antitumor Activity

The next study of *in vitro* antitumor activities of GEF was concentrated on the selected CH/β-GP hydrogel formula, as indicated in Figure 5. It is the best and had an acceptable GEF release

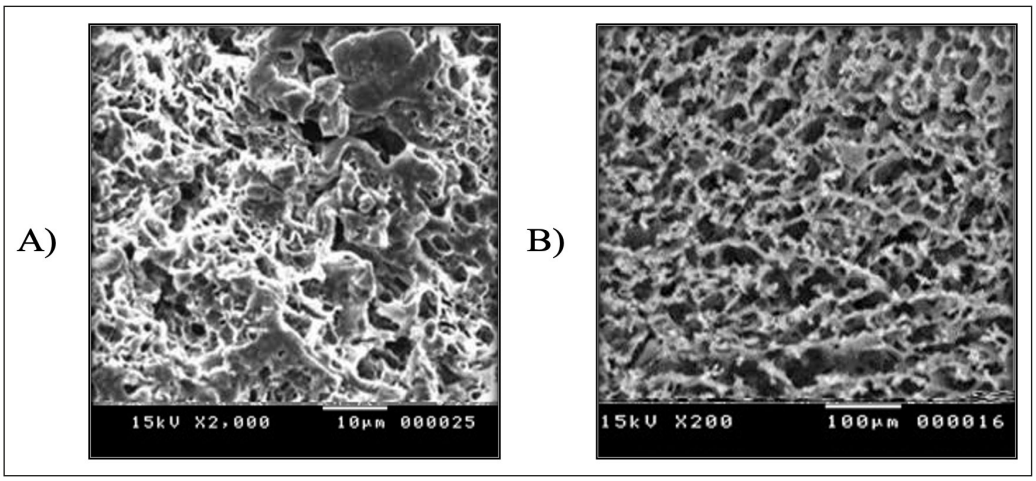

Figure 3. Morphological characterization of the medicated CH-based hydrogel systems; CH/β-GP (**A**) and CH/β-GP/Pl F127 (**B**) as shown by SEM.

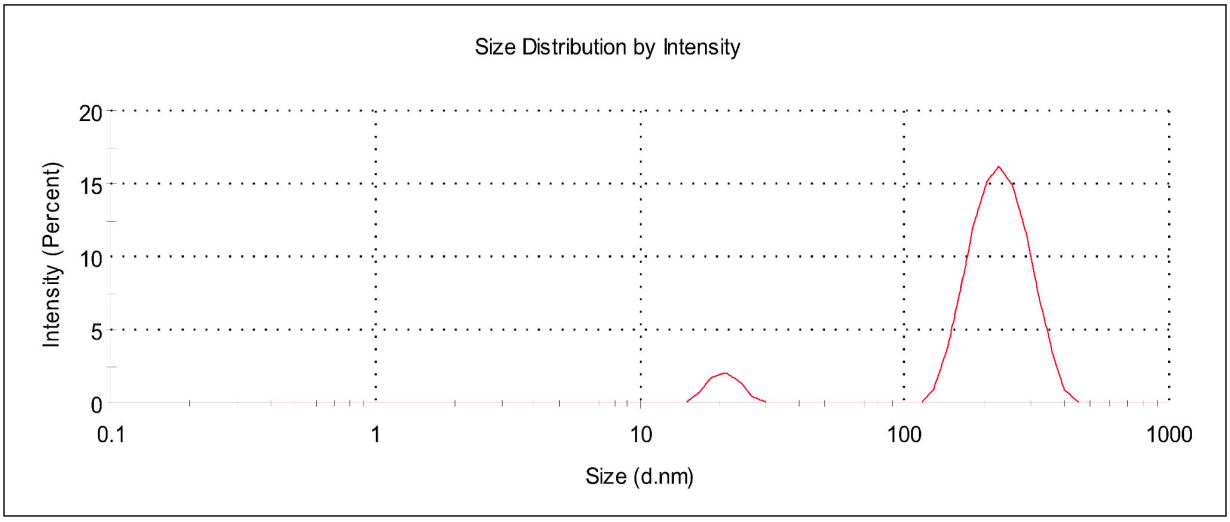

Figure 4. Particle size distribution of medicated CH-based hydrogel (CH/β-GP/Pl F127 type, as a representative example).

Table I. Kinetic data for percentage GEF released from CH/β-GP and CH/β-GP/Pl F127 hydrogel systems.

Kinetic models		Different kinetic orders			
Polymer-based hydrogel		1st	2nd	Higuchi diffusion	Korsmeyer-Peppas
CH/β-GP	r	0.922	0.851	0.975	0.968
	Slope/(n)	10.52	0.27	38.96	0.554
CH/β-GP/Pl F127	r	0.933	0.917	0.980	0.977
	Slope/(n)	6.65	0.05	25.2	0.568

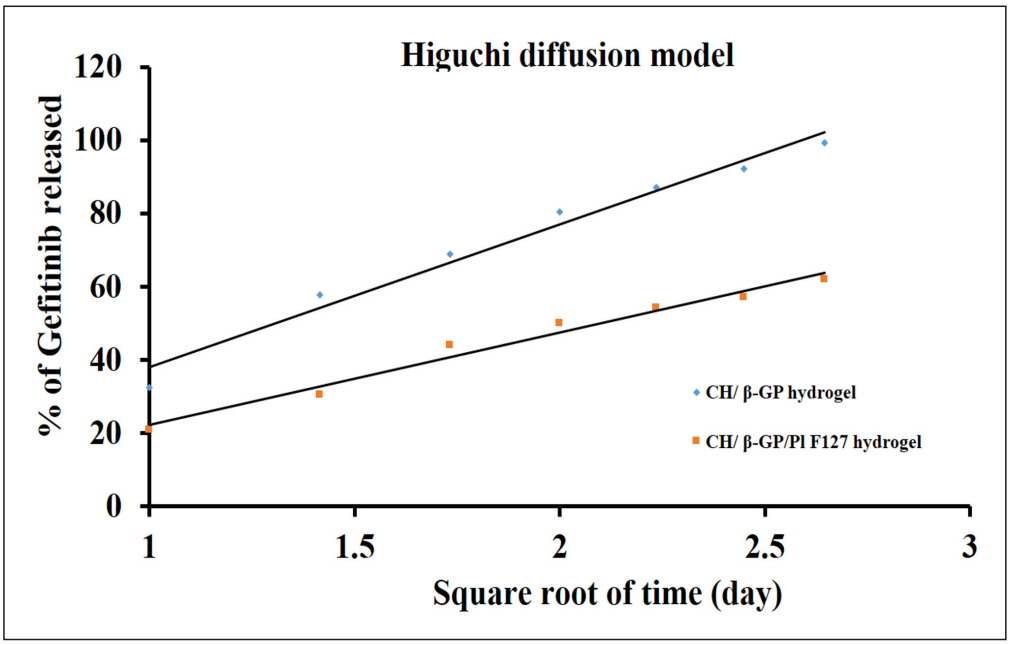

Figure 5. Kinetics release profiles of GEF from CH/β-GP and CH/β-GP/Pl F127 hydrogel systems plotted according to Higuchi's diffusion mechanism.

behavior. Thus, the antiproliferative effect of the proposed CH/β-GP hydrogel formula against the hepatic cancerous cell, HepG2, was investigated using the MTT tetrazolium salt colorimetric assay. The results of experimental trials (n = 3) treating HepG2 cells with GFB in saline solution as a positive control, free CH-based hydrogels as a negative control, and 0.5% GFB, w/v incorporated hydrogels at five different concentrations (6.25, 12.5, 25, 50 and 100 µg/mL), for 24 h, are shown in Table II and Figure 6, and were calculated using the following equations:

$$\%\ \text{of Cell Viability} = \left(\frac{\text{sample}-\text{blank}}{\text{control}-\text{blank}}\right) \times 100\%$$

$$\%\ \text{of Cell inhibation} = 1 - \left(\left(\frac{\text{sample}-\text{blank}}{\text{control}-\text{blank}}\right) \times 100\%\right)$$

The results revealed a significant difference between the viability of cells treated with free GFB and that treated with the medicated CH/β-GP hydrogel formula. HepG2 cells showed a distinct increase in the IC_{50} with GFB incorporated hydrogel systems (IC_{50} = 17.783 µg/mL of CH/β-GP formula) as compared against a free GFB (IC_{50} = 10.0 µg/mL).

Pharmacokinetic Studies

GEF concentrations in albino rats' plasma previously treated with medicated hydrogel solution were detected by a developed and validated LC method. The GEF concentration level showed a continued increase in rats' plasma during the first 4 days (T_{max}) with a plasma peak level (C_{max}) of 3.663 µg/mL after the injection of the medicated CH/β-GP hydrogel formula. However, this concentration decreased below the limit of detection within 15 days. Theoretically, these results can be explained because GEF goes through the network barriers and then diffuses into the circulation, thus prolonging its retention time inside the treated tissues. Moreover, the systemic exposure generated the mean residence time (MRT) of 9 days with ($AUC_{0\text{-}t}$) of 36.493 µg/mL/day. The obtained results indicated that the medicated injectable CH/β-GP hydrogel formula significantly increased the distribution of GEF within the tissues, leading to more excellent antitumor activity and lesser toxicity against the free GEF in saline when injected directly into the affected organs. On the other hand, as illustrated in Figure 7, the plasma samples (n = 3) pharmacokinetic characteristics of the CH/β-GP hydrogel formula were quite distinct from the free GEF solution.

Table II. % of cell inhibition of the prepared CH/β-GP hydrogel formula compared against free GEF applied for cancerous HepG2 cell line.

SN	Sample code	Blank	HepG2 (cancerous liver cells)								
			Free CH-based hydrogel (Negative control)			Free GEF in saline (Positive control)			0.5% GEF-incorporated hydrogel systems		
			A	B	C	A	B	C	A	B	C
1	100 µg		1.815	1.787	1.801	0.245	0.212	0.223	0.426	0.538	0.517
	Mean Abs.	**0.120**		1.801			0.226			0.494	
	% of cell inhibition			0%			93.69%			77.75%	
2	50 µg		1.815	1.787	1.801	0.356	0.368	0.388	0.626	0.584	0.612
	Mean Abs.	**0.120**		1.801			0.370			0.607	
	% of cell inhibition			0%			85.13%			71.03%	
3	25 µg		1.815	1.787	1.801	0.533	0.556	0.587	0.814	0.915	0.875
	Mean Abs.	**0.120**		1.801			0.558			0.868	
	% of cell inhibition			0%			73.94%			66.33%	
4	12.5 µg		1.815	1.787	1.801	0.866	0.854	0.918	1.276	1.394	1.32
	Mean Abs.	**0.120**		1.801			0.879			1.331	
	% of cell inhibition			0%			59.22%			27.96%	
5	6.25 µg		1.815	1.787	1.801	1.443	1.543	1.449	1.725	1.727	1.692
	Mean Abs.	**0.120**		1.801			1.478			1.715	
	% of cell inhibition			0%			27.03%			5.12%	

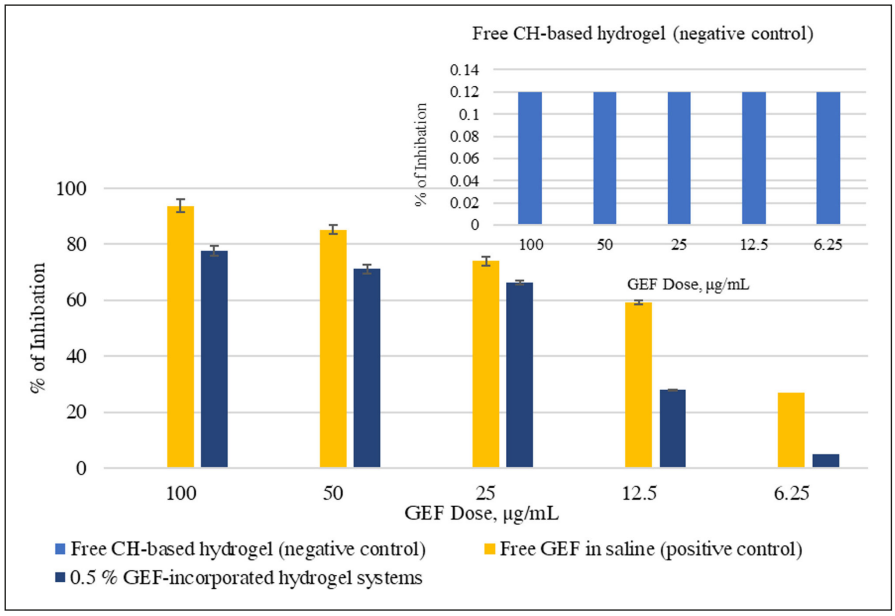

Figure 6. % of cell inhibition of the proposed CH/β-GP hydrogel formula compared with free GEF against HepG2 cancer cell line. Data are represented as mean ± S.D where n=3. There are no significant differences between the selected medicated hydrogel formula and the free form of GEF (the significant *p*-value = 0.350). At the same time, there are significant differences between the free form of GEF and its CH-based hydrogel formula, respectively at significant values of 0.0004 and 0.009.

Discussion

There are advantages accompanied by mixing some thermo-responsive polymers (namely CH and/or Pl F127) and a natural polysaccharide (β-GP) in getting a temperature-sensitive hydrogel[16]. The feature of the formed hydrogel can undergo a sol-gel phase transition between the room and body temperature, which is utilized as a smart device affected by the ambient condition or external stimuli at the administration site. Many factors could be used as stimuli, namely low pH, redox potential, and the presence of certain enzymes in case of disease states. Moreover, CH is considered an enzymatic, thermosensitive, and pH-sensitive polymer[16,42,43].

We confirmed this substantial difference in viscosity between the sol and gel phases due to a shift in the Unimer-micelle equilibrium, micellar development, or micellar expansion associated with an increase in aggregation number caused by hydrophobic or attractive forces[44].

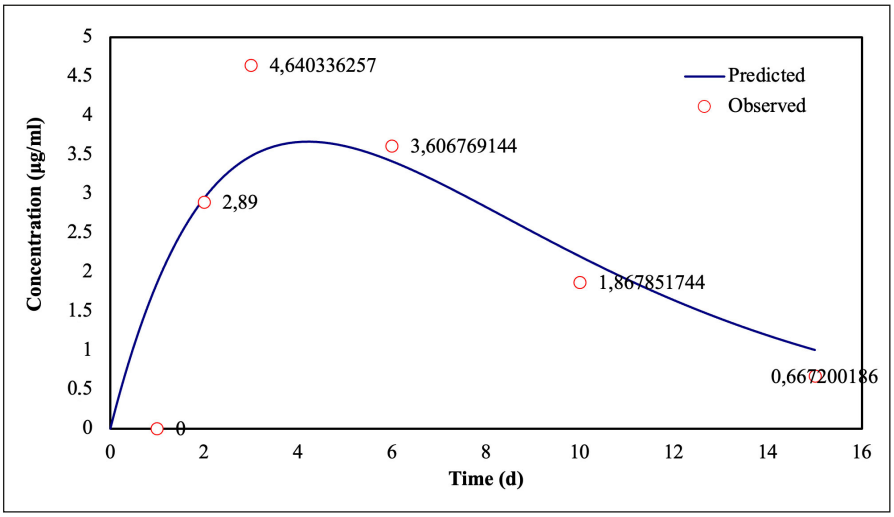

Figure 7. Concentration-time profiles of GFB at a dose of 25 mg/kg to healthy albino rat. GFB concentrations were determined by LC Agilent technologies 1200 series, G1321A FLD system using zorbax eclipse XDB-C18 column by isocratic elution. Each point represents the mean ± SD (n=4).

The results revealed that the pH was 6.5 and 6.7 for the medicated CH/β-GP and CH/β-GP/PL F127, which confirmed the utility of all formulations as subcutaneous injections, as the pH of injectable hydrogels was reported[45] to be between 6.5-7.4, to avoid the treated cell damage. Moreover, these medicated CH-based hydrogels showed a smooth and easy filling of the syringe with a size of (5 mm). The injection was extruded within 5-10 seconds and was pressed with fingers with mild to moderate force. The gelation point was measured for both systems to be found at 35.5 ± 0.5°C for the CH/β-GP system and 29.3 ± 1.5°C for CH/β-GP/Pl F127 system. According to these findings, the sol-gel transition occurred at body temperature as expected. There is a critical need for injectable hydrogels to offer a certain degree of syringeability and injectability[16]. Moreover, an acetic acid solution (0.1 M) could solubilize GEF and provide more than 10-fold sink conditions for *in vitro* release study. Accordingly, the enhanced solubility may be attributed to the adjustment of micro-environmental pH to modify the ionization behavior of GEF[46].

The higher viscosity of CH/β-GP/Pl F127 (120.8 ± 1.3 C_p ×10^3) system is caused by the presence of 20% of PI F127 in this system, as previously[47]confirmed. Fortunately, the obtained sustained release effect was intended to decrease a dosing frequency and provide a certain degree of similarity with chemotherapeutic protocols administered treatment[48].

Morphological analysis of polymeric systems provides an insight into their structure and organization. It could be used with an earlier mechanical and rheological evaluation of hydrogels[49]. The formulation's shape is frequently attributed to the interactions between the polar groups of the micellar copolymer and cellulose derivatives[50]. Both polymeric compositions generated a sponge-like structure, possibly linked to gel self-assembly as observed before for different PI F127 mixtures[51]. Thus, CH/β-GP-based hydrogel displayed an amorphous morphology. It is showed as a hydrophobic association like shape due to the hydrogen bonding between the polymers[51,52].

The behavior of the kinetic release model of GEF-incorporated hydrogel and dissolution studies were evaluated for 1st, 2nd orders models, Higuchi diffusion[29,36,53], and Korsmeyer-Peppas model[37,54]. The mathematical expression for different GEF release models was collected to select the most suitable model, based on the (correlation coefficient r) as suggested by Burnham and Anderson[38]. The drug release sequence was determined by the graphical depiction and validated by replacement[35,55,56]. The overall dissolution rate and GEF availability are controlled by the rate of GEF diffusion from the hydrogel layer, whatever its concentration is (which had the advantage of avoiding GEF-hydrogel characterized overload)[57-59]. On the other hand, the release data analysis by the Korsmeyer-Peppas model yielded values of n (release exponent) between 0.5 and 1.0 in both CH/β-GP and CH/β-GP/Pl F127 hydrogel systems, with non-Fickian release regulated by diffusion and chain relaxation processes[60].

We also confirmed a significant difference between the viability of cells treated with free GFB and that treated with the medicated CH/β-GP hydrogel formula. This result could be due to the improved solubility of GFB-incorporated hydrogel, which enhanced the cell penetration and potentiated its effect as an antiproliferative agent[61,62]. However, the outcomes exhibited less cytotoxic activities for the prepared CH/β-GP hydrogel formula, which suggest that the coating of GFB with the matrix of the hydrogel system leads to a barrier layer around its molecules. These findings indicate that the proposed CH-based hydrogel can be targeted into the type of solid tumor at pH 7.4, allowing its sustained dissolution and diffusion rates inside the solid mass of the tumor as compared to the free drug.

In the CH/β-GP hydrogel formula, the GFB concentration was maintained above the detection limit until 15 days which was consistent with the sustained release nature of its formula. The pharmacokinetic parameters indicated that the proposed hydrogel could be effectively used as a targeted GEF-carrier and facilitate its sustained release as indicated by the longer value of MRT of 9 days and an AUC_{0-t} of 41.917 μg/L/day. By comparing these results with that obtained from the cytotoxicity of the two formulations, we could see that the CH/β-GP hydrogel formula had a higher targeting efficiency for future solid tumor injection targeting than the free poor water soluble GFB. The expected results with tumor model rat would have a higher inhibition effect to a certain extent (Table III)[53].

Conclusions

A thermosensitive injectable CH-polymer-based hydrogel loaded with anticancer drug GEF was designed. Trials were conducted with a CH-based hydrogel system crosslinked with β-GP and Pl F127 to demonstrate the stability and efficacy

Table III. Distribution parameters of the medicated CH/β-GP hydrogel formula, after intratumor (i.t.) administration of 25 mg/kg GFB in the form of solution as a positive control and CH/β-GP hydrogel formula as the experiment into rats (n = 3).

Parameter	Unit	Value
$t_{1/2}k_a$	d	2.850
$t_{1/2}k_{10}$	d	2.986
V/F	(mg)/(μg/mL)	7.709
CL/F	(mg)/(μg/mL)/d	1.789
Tmax	d	4.209
Cmax	μg/mL	3.663
AUC_{0-t}	μg/mL*d	36.493
$AUC_{0-\infty}$	μg/mL*d	41.917
AUMC	μg/mL*d^2	352.949
MRT	d	8.420

Where k_a: absorption rate constants; F: absorption fraction; V: volume of distribution; $t_{1/2}k_{10}$ is the terminal half-life. The area under $C_{p/t}$ curve ($AUC0_{-t}$ and $AUC0_{-\infty}$) (ng × h/mL) was measured using the trapezoidal rule to indicate the extent of GEF absorption. The peak C_p (C_{max}). AUMC is the area under the concentration times time versus time curve. MRT is the mean residence time. Clearance, CL = Dose/AUC. T_{max} and $t_{1/2}$ (day) were estimated from Equation 1.

of the proposed method in terms of sustainability and antiproliferative effects with low systemic toxicity. The kinetics modeling of *in vitro* release studies revealed that the medicated CH/β-GP hydrogel formula was the best and with convenient properties. Further *in vitro* antitumor and *in vivo* pharmacokinetic modeling data were collected and interpreted on the side of the study. GFB-CH/β-GP hydrogel was considered a targeting and sustained device to perform intratumor injections to increase the retention time of GFB in plasma with a high value of C_{max} over 15 days in the laboratory rats. As a result, intratumor injection of GFB-CH/β-GP hydrogel system has the potential to be a successful targeted therapy approach for the future of solid tumors. Future study is needed to fully understand the anticancer impact of GFB-CH/β-GP hydrogel on solid tumors on pre- and clinical samples.

Availability of Data and Materials
The data supporting this study's results are available from the corresponding author.

Conflicts of Interest
The authors declare that they have no conflicts of interest regarding the publication of this article.

Ethics Approval
All animals were ethically approved for their experiments under the authority of the Faculty of Pharmacy, Al-Azhar University under number AZ-AS/PH/2/C/2021 and were maintained according to the Subcommittee of Health Research Ethics recommendations, Qassim University (Approval No.: 22-03-11), according to the National Research Council (US) Guide for the Care and Use of Laboratory Animals.

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Informed Consent
Not applicable.

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