Erzincan Üniversitesi
Fen Bilimleri Enstitüsü Dergisi
2022, 15(2), 636-648
ISSN: 1307-9085, e-ISSN: 2149-4584
Araştırma Makalesi

Erzincan University
Journal of Science and Technology
2022, 15(2), 636-648
DOI: 10.18185/erzifbed.1134093
Research Article

Preparation and Characterization of Chitosan/κ-Carrageenan Based Polymeric Nanoparticles for Gemcitabine Delivery

Habibe YILMAZ[1,2,3*]

[1]Pharmaceutical Biotechnology Department, Faculty of Pharmacy, Trakya University, 22030, Edirne, Turkey
[2]Biochemistry Department, Faculty of Science, Ege University, 35100, Izmir, Turkey
[3]Research and Application Center of Drug Development and Pharmacokinetics, Ege University, 35100, Izmir, Turkey.

Geliş / Received: 22/06/2022, Kabul / Accepted: 04/08/2022

Abstract

Cancer is the most fatal disease of the last century after cardiovascular disease. Gemcitabine is a nucleoside analogue used in the treatment of cancer. However, like many anticancer drugs, it has many side effects that limit treatment. A nano drug delivery system has been developed to provide effective treatment by reducing side effects. Chitosan and κ-carrageenan, which are regarded as safe by the FDA, were used in the preparation of the nano drug delivery system. In the synthesis, pre-ionic gelation followed by polyelectrolyte complexation method was used and then a second ionic gelation step was added. Chitosan: κ-carrageenan ratio and $CaCl_2$ concentration were optimized and the optimum polymer ratio was determined as 6:1 and $CaCl_2$ concentration was 2.5%. Its hydrodynamic size at optimum conditions was 393 nm and its size measured size in TEM was 20 nm. FTIR analyzes showed that nanoparticle synthesis was successful. Drug loading was performed by encapsulation and 58% drug loading was achieved. After drug loading, the hydrodynamic dimension was increased to 595 nm and its size measured size in TEM 45 nm. Drug release was monitored for 95 hours and was determined to be higher at pH 6.0 compared to 7.4 and pH sensitive. In addition, the Higuchi model is the most suitable mathematical model for drug release kinetics. The obtained results showed that chitosan: κ-carrageenan nanoparticles were suitable for gemcitabine delivery and were pH sensitive enough to respond to the tumor microenvironment.

Keywords: chitosan, kappa-carrageenan, gemcitabine, drug delivery, nanoparticle

Gemsitabin Salımı için Kitosan/κ-Karajenan Temelli Polimerik Nanopartiküllerin Sentezi ve Karakterizasyonu

Öz

Kanser, kardiyovasküler hastalıklardan sonra son yüzyılın en ölümcül hastalığıdır. Gemsitabin, kanser tedavisinde kullanılan bir nükleozit analoğudur. Ancak pek çok antikanser ilaç gibi tedaviyi sınırlayan pek çok yan etkisi mevcuttur. Yan etkileri azaltarak etkin tedavi sağlanması için nano ilaç taşıyıcı sistem geliştirilmiştir. Nano ilaç taşıyıcı sistem hazırlanmasında FDA tarafından güvenli tanımlanan kitosan ve κ-karajenan kullanılmıştır. Sentezde pre-iyonik jelasyon akabinde polielektrolit kompleksasyon yöntemi kullanılmış ve ardından ikinci bir iyonik jelasyon adımı eklenmiştir. Optimum koşullardaki hidrodinamik boyutu 393 nm ve TEM'de ölçülen boyutu 20 nm olarak ölçülmüştür. FTIR analizleri nanopartikül sentezinin başarılı olduğunu göstermiştir. İlaç yükleme enkapsülasyon yoluyla gerçekleştirilmiş ve %58 oranında ilaç yüklenmiştir. İlaç yüklemeden sonra hidrodinamik boyut 595 nm ve TEM ile ölçülen boyut 45 nm olarak ölçümlenmiştir. İlaç salımı 95 saat boyunca izlenmiştir ve pH 6,0'daki ilaç salımının 7,4'e kıyasla daha yüksek olduğu ve pH duyarlı olduğu belirlenmiştir. Ayrıca Higuchi modeli ilaç salım kinetiğini en uygun matematiksel modeldir. Elde edilen sonuçlar, kitosan:k-karajenan nanopartiküllerin gemsitabin taşımak için uygun olduğu ve tümör mikroçevresine yanıt verebilecek nitelikte pH duyarlı olduğu görülmüştür.

Anahtar Kelimeler: kitosan, kappa-karajenan, gemsitabin, ilaç taşınımı, nanopartikül

***Corresponding Author:habibeyilmaz@trakya.edu.tr**
Habibe YILMAZ, https://orcid.org/0000-0003-1106-0458

1. Introduction

Carrageenan (CRG) is a natural linear polysaccharide with a molecular weight of over 100 kDa, obtained from red seaweed (Rhodophyceae). It is obtained from species such *as Solieria* sp., *Eucheuma* sp., *Hypnea* sp., *Iridaea* sp., *Gigartina stellata, Chondrus crispus* and *Agardhiella* sp. It can be found in 6 forms as kappa (κ), lambda (λ), iota (ι), mu (μ), nu (ν) and theta (θ), depending on the number and position of sulphate groups in its structure [1]. It composed of linear chains of D-galactose and D-anhydrogalactose with ester sulphates. The most used forms of carrageenan are kappa, iota and lambda. κ-CRG has only one sulphate group, iota has two sulphate groups and lambda has three sulphate groups per disaccharide repeating unit which affects their charge density and solubility. The presence of Na^+, K^+, Ca^{2+} and Mg^{2+} ions and temperature were reported to be other parameters that affect solubility. Kappa and iota-CRG can form thermo-reversible gels. Anhydro bridges present in kappa and iota-CRG but not lambda-CRG have been suggested to influence gelation properties. Moreover, the $^1C_4$-conformation of the 3,6-anhydro-D-galactopyranosyl units provides the helical structures necessary for gel formation. They have high water absorption capacity, can form gels, contain negatively charged groups, form hydrophilic porous networks and can be cross-linked with other polymers or ions. Because of these properties, its use for sustained drug release, pH-sensitive drug delivery, temperature-sensitive drug delivery, tissue engineering, and drug dissolution improvement purposes is being investigated [2].

Chitosan (CS) is obtained by deacetylation of chitin. Sources of chitin are cell walls of fungi, exoskeletons of crustaceans and insects, scales of fish [3]. Chitosan is a pH-sensitive, biocompatible, cationic, mucoadhesive polysaccharide polymer composed of linear β-(1,4)-linked N-acetyl-D-glucosamine units and is FDA approved for tissue engineering and drug delivery use [4]. In addition to its antibacterial, antioxidant, analgesic and hemostatic properties, its biocompatibility and biodegradability and the non-toxicity of its degradation products have made it one of the ideal polymers in the field of biomedical application [5].

Gemcitabine (Gem) is a nucleoside analogue of deoxycytidine, which was initially used as an antiviral but was later used in solid tumors due to its effectiveness in the treatment of pancreatic cancer. It can be used as a single agent for first-line treatment in pancreatic cancer and in combination in the treatment of solid tumors such as colon, non-small cell lung cancer, breast, bladder and ovarian cancers. However, gemcitabine use is limited due to its rapid metabolism, development of drug resistance and toxic effects at high doses. For these reasons, it is necessary to develop new drug delivery systems for more efficient use [6].

Nanotechnology is utilized to deliver biologically active therapeutic ingredients to the targeted area. These drug delivery systems, which fall into the field of nanomedicine, can be polymeric, liposomal, polymeric micellar, metallic, dendrimer, etc. nanocarriers. These nano drug delivery systems can be passively or actively targeted to the relevant region. While the effect of enhanced permeation and retention (EPR) in the tumor region is utilized in passive transport, a more efficient transport can be achieved through the targeting ligand in addition to the EPR effect in active transport [7].

The aim of this study is to develop and characterize a nano drug delivery system for gemcitabine, which still needs to develop a new drug delivery system due to its poor pharmacokinetics. For this purpose, chitosan, and carrageenan, which are natural, biocompatible and biodegradable polymers known to have bioactive properties, were used.

2. Material and Methods

2.1. Materials

Dialysis tubing (12.000 MWCO), Tripolyphosphate (TPP), kappa-carrageenan, chitosan, supplied from Sigma Aldrich. Calcium chloride, supplied from MERCK. All other reagents were at analytical grade.

2.2. Synthesis of Chitosan/κ-Carrageenan Nanoparticles (CS/CRG NPs)

Different CS: κ-CRG and $CaCl_2$ ratios effect on CS/CRG nanoparticles were investigated. The combination of two different methods was used in the synthesis [8, 9].

1 mg/mL chitosan solution prepared in 1% acetic acid and 2.5 mg/mL κ-CRG solutions were mixed at room temperature in the ratios of 3.5:1, 4:1, 5:1, 6:1 and 7:1 (v/v). The mixture was allowed to mix for 30 minutes at a mixing speed of 300 rpm for polyelectrolyte complexation. Then, 10 mg/mL TPP added dropwise to the medium at a flow rate of 1 mL/min, with a 1:1 ratio of κ-CRG: TPP (v/v). After 30 minutes of reaction with TPP, 1% $CaCl_2$ solution was added at a flow rate of 1 mL/min, with a TPP: $CaCl_2$ ratio of 1:1 (v/v). 30 minutes post-addition of $CaCl_2$, nanoparticles were collected by centrifugation at 13000 rpm and washed 3 times with d.water.

To investigate the effect of using different $CaCl_2$ concentrations on nanoparticle formation and size, 1.5%, 2%, 2.5% and 3% concentrations were also investigated besides 1% concentration. At this stage, the experimental procedure explained above was applied after the optimum chitosan ratio was determined.

2.3. Characterization of CS/CRG Nanoparticles

Before determining the morphological structure, zeta size measurement was used in different CS: κ-CRG and $CaCl_2$ ratio experiments, and the optimum conditions were decided with the obtained data. Zeta size measurement was performed using the Malvern Zeta Sizer at Ege University Research and Application Center of Drug Development and Pharmacokinetics. Prior to measurement, samples were sonicated and dispersed in 3 x 3 minutes cycles. The refractive index of the material used in the measurement was 0.14 and the dispersant refractive index was 1.33.

FT-IR analysis was used for the structural confirmation of the nanoparticle. CS/CRG nanoparticles and, κ-CRG and CS as control were analyzed with FT-IR at Ege University Research and Application Center of Drug Development and Pharmacokinetics.

TEM imaging was used for morphological evaluation and performed at Çanakkale University ÇOBİLTUM using the JEOL JEM-1400 PLUS.

2.4. Synthesis of Gemcitabine Encapsulated CS/CRG NPs (Gem-CS/CRG NPs)

The drug encapsulation study was carried out under optimum nanoparticle synthesis conditions which were 6:1 CS: κ-CRG and %2.5 $CaCl_2$ concentration. The steps detailed above were used in nanoparticle synthesis. The only difference in drug loading is that gemcitabine is also added to the CS and k-CRG mixture before addition of TPP and further cross-linking with $CaCl_2$. Gemcitabine solution at concentrations ranging from 100 to 1000 µg/mL was used in the drug encapsulation study. Obtained nanoparticles were collected by centrifugation at 13000 rpm and washed 3 times with d. water. The determination of gemcitabine in the supernatants after precipitation and washing was performed spectrophotometrically (270 nm). The encapsulation efficiency (EE) was calculated using the following equation.

$$\text{EE}\ (\%) = \frac{\text{Initial Gem amount}(\mu g) - \text{Gem amount in supernatant}\ (\mu g)}{\text{Initial Gem amount}\ (\mu g)} \quad (1)$$

TEM used for morphological evaluation and FT-IR analysis used for structural evaluation.

2.5. *In vitro* Drug Release Study and Kinetics Evaluation

The nanoparticles were dispersed 10 mM acetate buffer at pH 6.0 and 10mM phosphate buffer at pH 7.4 and sealed in dialysis tubing (12.000 MWCO). The dialysis tubings were placed in relevant buffer containing vessels and then set in a water bath at 37°C with constant shaking. Samples were taken at predetermined time intervals and replaced with pre-warmed fresh media. The amount of Gem released in collected sample was measured spectrophotometrically at 270 nm. The cumulative fraction of released Gem versus time was expressed by the following equation.

$$\text{Cumulative Drug Release}\ (\%) = \text{Released Gem}\ (\mu g) \Big/ \text{Initial Gem amount}(\mu g) \quad (2)$$

Zero-order release, first-order release, Higuchi, Korsmeyer–Peppas, and Hixon–Crowell mathematical models were used to evaluate the data to determine drug release kinetics behavior. Correlation coefficient (R^2) value was used in the evaluation of the model. The highest R^2 value indicates the best-fit model [10].

All studies performed in triplicate and results were expressed as mean ± SD.

3. Results and Discussion

3.1. Synthesis and Characterization of Chitosan/κ-Carrageenan Nanoparticles (CS/CRG NPs)

A modification of the ionic pre-gelation/polyelectrolyte complexation method was used for the synthesis of nanoparticles. In the modified method, size control was achieved by adding an additional ionic gelation step to the ionic pre-gelation/polyelectrolyte complexation.

Initially, ionic pre-gelation was performed by cross-linking the amino groups of CS with TPP in the mixture of CS and κ-CRG. Therewithal, polyelectrolyte complexation occurred between the free amino groups of CS and the sulfated ester groups of κ-CRG. In addition, sulfated ester groups that did not participate in the complexation were cross-linked with $CaCl_2$ and subjected to a second ionic gelation process. Thus, the stabilization of the structure and the reduction of the size were ensured. Since the parameters that are effective in ionic gelation and polyelectrolyte complexation are polymer ratios and crosslinker concentration, these two parameters have been optimized [11]. Hydrodynamic size measurement and polydispersity index (PDI) were used during the optimization of the parameters. Optimization study data are shown in Table 1.

Table 1. Size and PDI values in different conditions.

Parameter	**Variation**	**Size (nm)**	**PDI**
CS: κ-CRG ratio	3.5:1	2822 ± 230.8	0.8667 ± 0.1623
	4:1	2472 ± 148.4	0.8526 ± 0.1777
	5:1	2173 ± 165.1	0.7422 ± 0.1813
	6:1	**1072 ± 148.4**	**0.622 ± 0.0983**
	7:1	1284 ± 155.8	0.3922 ± 0.1682
$CaCl_2$ (%)	1	833.7 ± 196.8	0.8275 ± 0.1196
	1.5	535. 6 ± 149.7	0.4454 ± 0.0896
	2	238.9 ± 96.4	1.4137 ± 0.1019
	2.5	**392.6 ± 9.6**	**0.3396 ± 0.0453**
	3	512.0 ± 58.3	0.4319 ± 0.0297

It is known that the gelation of κ-CRG occurs in the temperature-dependent transition from the coil to the helix conformation and the subsequent aggregation of the helices. The formed aggregates are stabilized by monovalent or divalent cations [12]. CS nanoparticles, on the other hand, can be synthesized by cross-linking the amine groups of chitosan protonated in a slightly acidic environment with TPP [13]. Polyelectrolyte complexation occurs as a result of electrostatic interaction of oppositely charged polyionic polymers. Among the factors that affect the formation of polyelectrolyte complexes are ion site, charge density, polyelectrolyte concentration, pH, ionic strength, solvents and temperature. It is stated that the modulation of these parameters is important in the control of the polyelectrolyte complexation process [14].
Triwulandari et al. were synthesized nanoparticles by polyelectrolyte complexation method using chitosan and k-CRG, TPP and SDS as polyanions. In the study, they showed that a 6:1:1 ratio of CS:k-CRG:TPP resulted in nanoparticles of approximately 750 nm in size [15]. Dimensions obtained in the study Triwulandari et al. higher than they achieved. The size difference may occur since the polymers used may have different batch numbers, different molecular weight distribution, and different degrees of acetylation for chitosan.

In the study of Rodrigues et al., the CS: κ-CRG: TPP ratio was evaluated in terms of nanoparticle formation and size. They reported that the synthesis resulted in nanoparticle

formation, precipitation, or remaining as solution, since the polymer ratios affect the +/- charge distribution [9]. On the other hand, in this study, it has been shown that the size can be further reduced by additional ionotropic gelation step to the ionotropic pre-gelation/polyelectrolyte complexation method. The hydrodynamic size was approximately 393 nm and, the size obtained from the TEM analysis was determined approximately 20 nm as can be seen from Figure 1A. According to the TEM image obtained from the study of Rodrigues et al., the nanoparticle sizes are approximately 400 nm and are 20 times larger. These data demonstrate that the addition of a secondary ionotropic gelation step to the ionotropic pre-gelation/polyelectrolyte complexation method is effective in size control.

PDI values were also considered in determining the optimum nanoparticle synthesis conditions. While the size was evaluated at the polymer ratio stage, both the size and PDI value were evaluated together at the crosslinker concentration stage. The final PDI value obtained is between 0.1 and 0.4. As stated in Bhattacharjee's article, the PDI value of 0.1 – 0.4 are moderately polydisperse. [22]. As Webber et al. stated in their article, natural k-carrageenan has a very high molecular weight and polydispersity. However, the addition of ions such as K^+ in high concentration to the medium reduces the interchain repulsion, thus giving a more folded and compact structure, while reducing the polydispersity index [23]. Therefore, while the polydispersity index was high in the polymer ratio study, the PDI value decreased in the $CaCl_2$ concentration study at the concentration that modulates the repulsion appropriately for optimum folding. However, still, different molecular weights and chain lengths in the polymer hinder the formation of a completely monodisperse nanoparticles.

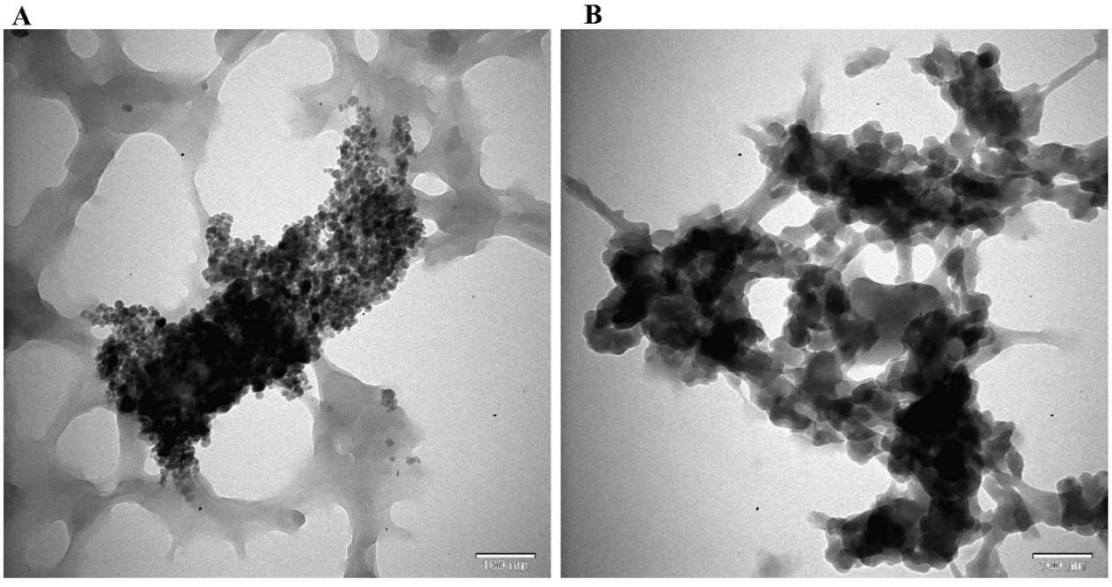

Figure 1: TEM images of A) CS/CRG NPs and B) Gem-CS/CRG NPs. (Scale bar is 100 nm).

The peaks at 3396 cm^{-1} are observed belong to the O-H stretch in κ-CRG. The peak at 2900 cm^{-1} belongs to $-CH_2$ groups in both polymers. The peaks at 842 cm^{-1} and 923 cm^{-1} in the FT-IR fingerprint region are characteristic sulphate group signals. Peaks at 1124 cm^{-1} and 1157 cm^{-1} belongs to C-O-C which is attributed to 3,6-anhydro-D-galactose.

Signals at 3300 cm^{-1} and 3367 cm^{-1} are obtained due to the primary amine and hydroxyl groups of chitosan. C-O stretching at 2879 cm^{-1}, N-H at 1654 cm^{-1}, CN at 1419 cm^{-1} and C-O-C signals at 1064 cm^{-1} confirm the structure of chitosan.

In nanoparticles, signals from the amino group of chitosan and the sulfate groups of κ-CRG are suppressed. This finding confirms that the respective groups form nanoparticles via ionic gelation/polyelectrolyte complexation as expected. On the other hand, signals recorded at 2322 cm^{-1}, 2341 cm^{-1} and 2343 cm^{-1} due to C=N stretch in gemcitabine were recorded only at 2322 cm^{-1}, 2339 cm^{-1} and 2343 cm^{-1} in drug-loaded nanoparticle. It is seen that the $-NH_2$ signal in gemcitabine, seen at 3387 cm^{-1}, is lost in the nanoparticle. This data shows that the amino group of gemcitabine interacts electrostatically with κ-CRG in the polyelectrolyte complexation step and is thus encapsulated.

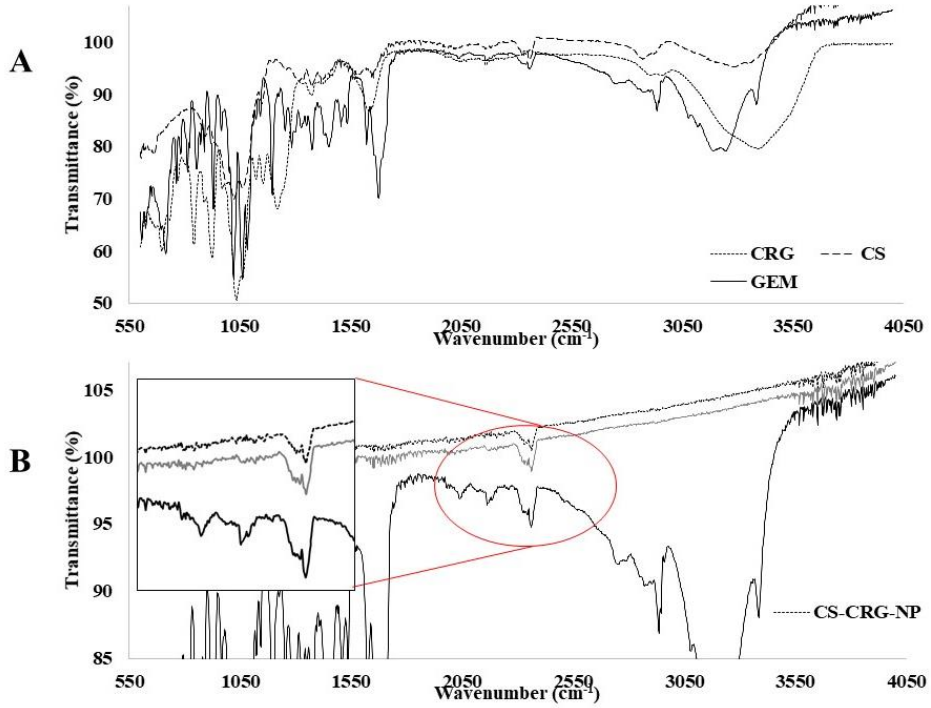

Figure 2: FTIR analysis spectrums of A) Initial materials that are CS, CRG and GEM and, B) Gem, CS/CRG NPs and Gem-CS/CRG NPs.

3.2. Synthesis of Gemcitabine Encapsulated CS/CRG NPs (Gem-CS/CRG NPs)

Prior to encapsulation for drug loading, the absorption approach was attempted. However, since the adsorption efficiency was around 2% (data not shown). It was concluded that the failure of the adsorption approach was because most of the groups that would interact with Gemcitabine, as seen in the FTIR analysis, participate in the ionic gelation/polyelectrolyte complexation process and there is no free group left. Therefore, the encapsulation method was preferred.
Gemcitabine determination was carried out spectrophotometrically at 270 nm wavelength using the $y = 0.0157x + 0.0012$ (R^2=0.9997) equation calculated from calibration curve.

As can be seen in Figure 3, during encapsulation of CS/ κ-CRG nanoparticles, the encapsulation efficiency was found around 58% for 0.75 mg/mL drug concentration, due to the interaction of sulphate groups of κ-CRG with gemcitabine. However, the lower efficiency compared to other polymeric nanoparticles was thought to be due to the electrostatic complexation of CS/κ-CRG polymers to form nanoparticles with each other, as well as with gemcitabine. Yew and Misran encapsulated different active ingredients such as ascorbic acid, caffeine and lidocaine into the nanoparticles prepared with CS and k-CRG. The encapsulation efficiencies were determined as 72%, 28% and 17% for ascorbic acid, caffeine, and lidocaine, respectively. They commented that this difference in encapsulation efficiencies is due to the solubility of the active ingredients and the repulsion power due to their charge [16].

The hydrodynamic dimension after loading the drug was determined as 565.3 ± 38.8 and the PDI value as 0.9228 ± 0.0045. The PDI value obtained from hydrodynamic size measurement shows that uniformity decreases after gemcitabine encapsulation. As seen in Figure 1B, after gemcitabine encapsulation, its dry size increased to approximately 45 nm. This change is because some of the sulphate groups of κ-CRG interact with gemcitabine during complexation and cannot be as tightly packed with chitosan as it is in the non-encapsulated state.

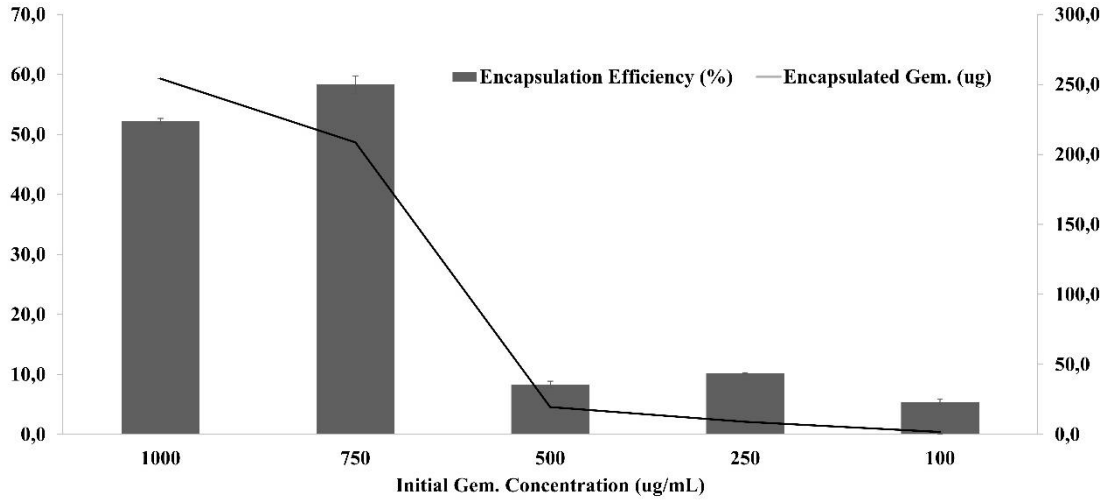

Figure 3: Encapsulation efficiency and amount of gemcitabine into CS/-κ-CRG NPs.

3.2. *In vitro* Drug Release Study and Kinetics Evaluation

The drug release study was performed in duplicate at pH 6.0 mimicking the tumor microenvironment and 7.4 as physiological pH for 95 h. As seen in Figure 4A, drug release was higher at physiological pH in the first 5 hours. At the 5th hour, 35.8% of gemcitabine release was detected at pH 7.4 and 32.9% at pH 6.0. However, as of the 7th hour, the release amount at pH 6.0 was higher than at physiological pH and reached 100% while pH 7.4 was 93.1% at the 95th hour. According to these results, it can be suggested that the drug delivery system has a controlled and sustained drug release profile in pH sensitive manner.

Ovalbumin was loaded into CS/CRG nanoparticles synthesized by [8]. The drug release trial was performed only at pH 7.4 and it was determined that all ovalbumin was released in 21 hours. They reported that the release at the relevant pH in the first 5 hours was around 18% and the burst effect was not observed. In the study, the drug release at the relevant pH was 39.1% at the 21st hour and 93% at the end of the 95th hour. This shows that drug release produces approximately 5 times longer and controlled drug release in the drug delivery system synthesized by Grenha et al [8]. In the study carried out by Wathoni et al. in 2021, chitosan nanoparticles were synthesized via ionic gelation with TPP and α-mangostin was encapsulated [17]. κ-CRG, on the other hand, was added to the structure by coating the surface of these nanoparticles with the solvent evaporation method. Drug release was studied in two different environments, pH 1.2 and 7.4. In the first 5 minutes, 34% and 42% release were observed for pH 1.2 and 7.4, respectively. After the burst effect was seen in the first 5 min., the drug release was reached to %60 in 60 minutes. In the study carried out, the drug release at the 1st hour was 26.3% for pH 7.4 which is 2-fold lower than the study of Wathoni et al [17].
Five different mathematical models were studied to determine the behavior of drug release. Among the 5 kinetic models, the model with the most correlation of drug release is the Higuchi model as can be seen from Table 2 and Figure 4B.

Table 2. Regression equation, rate constant (k) and correlation coefficients of the studied mathematical models.

Model	pH	Regression equation	Rate Constant (k)	R^2
Zero-order	6.0	1.0211x + 26.594	1.0211	0.7126
	7.4	0.7922x + 28.649	0.7922	0.7709
First-order	6.0	-0.0118x + 1.96	0.0271	0.9796
	7.4	-0.0091x + 1.9655	0.0209	0.9670
Higuchi	6.0	10.95x – 1.1849	12.415	0.9930
	7.4	9.1384x – 0.795	10.988	0.9820
Korsmeyer-Peppas	6.0	0.7885x + 6845	17.4042 (n=0.41)	0.7689
	7.4	0.7495x + 0.653	21.1109 (n=0.31)	0.7678
Hixon–Crowell	6.0	0.0551x + 0.1689	0.0584	0.9874
	7.4	0.0255x + 0.4259	0.0338	0.9295

The zero order model states that the drug release is only time dependent, and the release occurs very slowly. The R^2 values for both pHs' in the table show that the drug release mechanism of the nano drug delivery system cannot be explained by this model. In other words, time is not the only parameter that is effective in drug release. The first-order model, on the other hand, suggests that the time-dependent change in drug concentration is only concentration-dependent. Although the R^2 value is greater than 0.95, it does not fully explain the drug release mechanism

when compared to other models. However, it is possible to state that one of the effective factors in drug release is concentration. The Hixson-Crowell model describes systems in which the surface area and diameter of the drug matrix change over time. The R^2 values obtained in this model differ for pH 6.0 (R^2 = 0.9874) and 7.4(R^2 = 0.9295). This data can be evaluated that the surface area and diameter of the drug matrix change with time at pH 6, whereas such a change is less at pH 7.4. This situation can be explained by the fact that the ionizable groups of the drug carrier matrix have different charges at the relevant pHs' depending on the pKa values, the repulsion forces are different and the change in diameter is therefore different. Korsmeyer-Peppas is mostly used for drug matrices such as hydrogels. Since the R^2 value obtained from the model is quite low, drug release cannot be explained by this model. As a matter of fact, it can be said that the obtained structure does not have swelling characteristics like hydrogels. However, the n value obtained was 0.41 for pH 6.0 and 0.31 for pH 7.4, which suggested that the release was more suitable for Fickian diffusion. However, this value cannot be said to be completely reliable due to the low regression coefficient of the model. When analyzed in terms of k values, the constants at pH 6.0 are higher than those at pH 7.4, indicating that the release at pH 6.0 is faster than pH 7.4, and it releases more drug per unit time.

The first mathematical model of drug release from matrix system was introduced by Higuchi. It can be used for the release of water-soluble drugs from polymeric matrices. It is based on the hypothesis that i) the drug concentration in the matrix is higher than the solubility of the drug, ii) the diffusion is unidirectional, iii) the drug particle size is smaller than the matrix thickness, iv) the matrix swelling, and dissolution is negligible, v) the drug diffusivity, and vi) perfect sink conditions are always attained in the release medium [18]. According to the obtained data, it can be concluded that the drug release is dependent on time and drug concentration, and the swelling or dissolution of the nanoparticle is not negligible. When the Higuchi model and drug release graphs are examined, there is almost no difference in the amount of release between pH 6.0 and 7.4. It is thought that the solvent gradually diffuses into the polymer matrix during this time. However, after the 21st hour, while the solvent gradually diffuses into the polymer matrix, it is suggested that the pH of the solvent has a greater effect on the ionizable groups of the polymer matrix after this period. In fact, the different R^2 values of the mathematical model at pH 6.0 and pH 7.4 show that the system is pH sensitive. This can be explained by the fact that the polymer contains ionizable groups and is therefore affected by pH. Inter- and intra-molecular interactions between functional groups of the polymer are important during preparation of porous nanoparticles [19]. The amino groups of CS are partially protonated at acidic pH, which facilitates its interaction with both k-CRG and gemcitabine. On the other hand, when the pH increases, the amino groups deprotonate and their interaction decreases [20], which reduces the interaction with the drug and polymer, causing an increase in the size of the pores in the nanoparticle. Besides, sulphate groups in k-CRG are negatively charged at pHs above the pKa value [21]. As the negative charge increases, the pore size increases due to repulsion. As the pH value increases, it is expected that the repulsion power will increase, and the pore size will increase due to the increase in the amount of negative charge. Therefore, as the pH value increased, more water molecules could diffuse, and the drug release was higher compared to pH 6.0. This situation reveals that diffusion is the driving force and pore diameter

is an effective parameter, as explained in the Higuchi model. It also reveals that the system is pH sensitive.

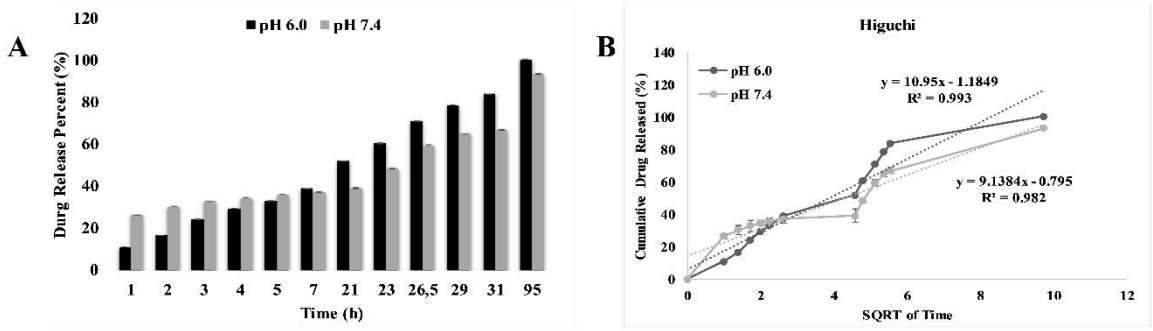

Figure 4. A) Drug release profile of GEM-CS-κ-CRG at pH 6.0 and 7.4 **B)** Higuchi drug release kinetics model.

4. Conclusion

In the study, a pH-sensitive controlled drug delivery system was developed for gemcitabine using chitosan and kappa-carrageenan. Nanoparticles were obtained by combination of polyelectrolyte complexation and ionic gelation methods using chitosan and κ-CRG, which are regarded as safe by FDA. After the chitosan nanoparticles were synthesized with TPP cross-linker via ionic gelation, polyelectrolyte complexation was performed by adding k-CRG to the medium. However, due to the high molecular weight of both polymers, the hydrodynamic dimensions are expected to be large. To reduce the dimensions, another ionic gelation step was added by crosslinking the k-CRG with $CaCl_2$ after the polyelectrolyte complexation. The added second ionic gelation step resulted in a reduction in size as expected. Thus, it has been shown that adding a second ionic gelation step to this approach, which is known as the ionotropic pre-gelation/polyelectrolyte complexation method in the literature, is beneficial in size control. Drug loading was successful with a 58% yield at an initial of 0.75 mg/mL of gemcitabine concentration. The pH sensitivity of the resulting drug delivery system was investigated in the drug release study since the tumor microenvironment pH may be as low as pH 6.0. It has been observed that the system is pH sensitive in drug release. The mathematical model that best explains drug release kinetics was Higuchi. In other words, diffusivity and drug concentration affect drug release. The sustained, controlled and pH-sensitive drug release of the synthesized nanoparticles suggests that it is a potential drug delivery system for gemcitabine.

Ethics in Publishing

There are no ethical issues regarding the publication of this study.

Author Contributions

Habibe YILMAZ is the sole author who designed and executed the study, collected, and analyzed the data and prepared the article.

Acknowledgements

I acknowledge Prof. Dr. Şenay Hamarat Şanlıer for allowing me to use her laboratory equipment and consumables.

References

[1] Akbar, A. and Shakeel A., (2019). Marine Polysaccharides. Shakeel A. and Aisverya S. (Eds.), Carrageenans: Structure, Properties and Applications (pp. 29-52). Jenny Stanford Publishing, USA.

[2] Liu, J., Zhan, X., Wan, J., Wang, Y., & Wang, C. (2015). Review for carrageenan-based pharmaceutical biomaterials: Favourable physical features versus adverse biological effects. *Carbohydrate Polymers, 121*, 27-36.

[3] Yanat, M., & Schroen, K. (2021). Preparation methods and applications of chitosan nanoparticles; with an outlook toward reinforcement of biodegradable packaging. *Reactive & Functional Polymers, 161*.

[4] Mohammed, M., Syeda, J., Wasan, K., & Wasan, E. (2017). An Overview of Chitosan Nanoparticles and Its Application in Non-Parenteral Drug Delivery. *Pharmaceutics, 9*(4).

[5] Muxika, A., Etxabide, A., Uranga, J., Guerrero, P., & de la Caba, K. (2017). Chitosan as a bioactive polymer: Processing, properties and applications. *International Journal of Biological Macromolecules, 105*, 1358-1368.

[6] Paroha, S., Verma, J., Dubey, R., Dewangan, R., Molugulu, N., Bapat, R., et al. (2021). Recent advances and prospects in gemcitabine drug delivery systems. *International Journal of Pharmaceutics, 592*.

[7] Patra, J., Das, G., Fraceto, L., Campos, E., Rodriguez-Torres, M., Acosta-Torres, L., et al. (2018). Nano based drug delivery systems: recent developments and future prospects. *Journal of Nanobiotechnology, 16*.

[8] Grenha, A., Gomes, M., Rodrigues, M., Santo, V., Mano, J., Neves, N., et al. (2010). Development of new chitosan/carrageenan nanoparticles for drug delivery applications. *Journal of Biomedical Materials Research Part a, 92A*(4), 1265-1272.

[9] Rodrigues, S., da Costa, A., & Grenha, A. (2012). Chitosan/carrageenan nanoparticles: Effect of cross-linking with tripolyphosphate and charge ratios. *Carbohydrate Polymers, 89*(1), 282-289.

[10] Ak, G., Bozkaya, U., Yilmaz, H., Turgut, O., Bilgin, I., Tomruk, C., et al. (2021). An intravenous application of magnetic nanoparticles for osteomyelitis treatment: An efficient alternative. *International Journal of Pharmaceutics, 592*.

[11] Patil, J., Marapur, S., Gurav, P., Banagar, A., & Mishra, M. (2016). Ionotropic Gelation and Polyelectrolyte Complexation Technique Novel Approach to Drug Encapsulation. *Handbook of Encapsulation and Controlled Release*, 273-296.

[12] Daniel-da-Silva, A. L., Ferreira, L. u., Gil, A. M., & Trindade, T. (2011). Synthesis and swelling behavior of temperature responsive κ-carrageenan nanogels. *Journal of Colloid and Interface Science, 35*(2), 512-517.

[13] Pedroso-Santana, S., & Fleitas-Salazar, N. (2020). Ionotropic gelation method in the synthesis of nanoparticles/microparticles for biomedical purposes. *Polymer International, 69*(5), 443-447.

[14] Lankalapalli, S., & Kolapalli, V. (2009). Polyelectrolyte complexes: A review of their applicability in drug delivery technology. *Indian Journal of Pharmaceutical Sciences, 71*(5), 481-487.

[15] Triwulandari, E., Fahmiati, S., Sampora, Y., Meliana, Y., Ghozali, M., Sondari, D., et al. (2018). Effect of Polyanions Variation on The Particle Size of Chitosan Nanoparticle

Prepared by Ionic Gelation Method. *Proceedings of the 4th International Symposium on Applied Chemistry 2018, 2024*.

[16] Yew, H.-C., & Misran, M. (2016). Preparation and characterization of pH dependent κ-carrageenan-chitosan nanoparticle as potential slow release delivery carrier. *Iranian Polymer Journal, 25*, 1037-1046.

[17] Wathoni, N., Meylina, L., Rusdin, A., Abdelwahab Mohammed, A.F., Tirtamie, D., Herdiana, Y., Motoyama, K., Panatarani, C., Joni, I.M., Lesmana, R., Muchtaridi, M. (2021). The Potential Cytotoxic Activity Enhancement of α-Mangostin in Chitosan-Kappa Carrageenan-Loaded Nanoparticle against MCF-7 Cell Line. *Polymers*, 13(11), 1681 - 1694.

[18] Dash, S., Murthy, P.N., Nath, L., Chowdhury, P. (2010). Kinetic Modeling on Drug Release from Controlled Drug Delivery System. *Acta Poloniae Pharmaceutica - Drug Research*, 67(3), 217-223.

[19] Amgoth, C., Phan, C., Banavoth, M., Rompivalasa, S., & Tang, G. (2019). Polymer Properties: Functionalization and Surface Modified Nanoparticles. In R. K. Tyagi, N. Garg, R. Shukla, & P. S. Bisen (Eds.), Role of Novel Drug Delivery Vehicles in Nanobiomedicine. IntechOpen. https://doi.org/10.5772/intechopen.84424.

[20] Sogias, I.A., Khutoryanskiy, V.V., Williams, A.C. (2010). Exploring the Factors Affecting the Solubility of Chitosan in Water. *Macromolecular Chemistry and Physics*, 211(4), 426 - 433.

[21] Distantina, S., Rochmadi, Fahrurrozi, M. Wiratni. (2014). Stabilization of Kappa Carrageenan Film by Crosslinking: Comparison of Glutaraldehyde and Potassium Sulphate as the Crosslinker. *2014 5th International Conference on Chemical Engineering and Applications*, 74, 5pp.

[22] Bhattacharjee, S. (2016). DLS and zeta potential - What they are and what they are not?. *Journal of Controlled Release*, 235, 337-351.

[23] Webber, V., de Carvalho, S., & Barreto, P. (2012). Molecular and rheological characterization of carrageenan solutions extracted from Kappaphycus alvarezii. *Carbohydrate Polymers*, 90(4), 1744-1749.