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Research Article

Alginate/Chitosan Nanoparticles for Adsorption and Controlled Release of Pemetrexed

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Abstract

Pemetrexed (PEM) is used for treatment of non-small cell lung cancer. However, PEM has disadvantages like fast elimination, low bioavailability, poor tumor cell selectivity, and penetration. Thus, there is a need for using pemetrexed delivery system to increase the anticancer effect of drug in lung cancer cells and to minimize its side effects. The purpose of this study is development of alginate/chitosan nanoparticles (ACNP) that have biodegradable and non-toxic structure for effective delivery of PEM for lung cancer therapy. In the present study, ACNP were prepared using the ionic gelation method, and pemetrexed was loaded via the adsorption method. Drug adsorption efficiency was calculated to be 57.80% and characterization studies were performed. *In vitro* drug release tests were carried out at pH levels of 5.5 and 7.4 with pemetrexed-loaded alginate/chitosan nanoparticles (PACNP) and free pemetrexed, and both the results were subsequently compared. Up to 11% release yield was observed at pH 5.5, and the yield reached up to 7% in pH 7.4 in the 25 hours. This nanoparticle system could be investigated *in vitro* and *in vivo* in further studies for controlled release of pemetrexed.

Keywords: *Alginate/chitosan nanoparticle, pemetrexed, adsorption, lung cancer, passive targeting*

Aljinat/Kitosan Nanopartiküllere Pemetrekset Adsorpsiyonu ve Kontrollü İlaç Salımı

Öz

Pemetrekset (PEM) küçük hücre dışı akciğer kanseri tedavisi için kullanılmaktadır. Bununla birlikte, pemetreksetin hızlı eliminasyon, düşük biyoyararlanım, zayıf tümör hücresi seçiciliği ve penetrasyon gibi sorunları vardır. Pemetreksetin akciğer kanseri hücrelerinde etkisini artırmak ve yan etkilerini en aza indirmek için ilaç taşıyıcı sistem kullanımına ihtiyaç vardır. Bu çalışmadaki amaç, biyobozunur ve non-toksik yapıdaki aljinat/kitosan nanopartiküllerine (ACNP) pemetrekset adsopsiyonunu gerçekleştirerek, pemetreksetin vücut içinde etkin bir şekilde taşınımını ve istenilen bölgede salımını sağlamaktır. Çalışmada iyonotropik jelasyon yöntemi kullanılarak aljinat/kitosan nanopartikülleri sentezlenip adsorpsiyon metodu ile pemetrekset taşıyıcı sisteme yüklendi. İlaç yükleme verimi % 57,80 olarak hesaplandı ve karakterizasyon testleri yapıldı. Pemetrekset yüklü aljinat/kitosan nanopartiküller (PACNP) ve serbest pemetrekset çözeltisi kullanılarak pH 5,5 ve 7,4 ortamlarında ilaç salım çalışmaları gerçekleştirildi. Elde edilen sonuçlar birbiriyle karşılaştırıldı. İlaç salım profili 25 saat boyunca izlendi ve nanopartiküllerden pH 7,4'te gözlenen %7'lik salım miktarının pH 5,5'ta %11'e yükseldiği belirlendi. Geliştirilen bu nanopartikül sistemi, pemetreksetin kontrollü salımı için daha ileri çalışmalarda in vitro ve in vivo olarak araştırılma potansiyeline sahiptir.
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Anahtar Kelimeler: *Aljinat/kitosan nanopartiküller, pemetrekset, adsorpsiyon, akciğer kanseri, pasif hedefleme*

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1. Introduction

Globally, cancer is the second leading cause of death, and according to the data World Health Organization, 19.3 million new cases and 10.0 million cancer-related deaths were reported in 2020. Lung cancer is a cancer type starting in the trachea, bronchia, or lung tissue and is most commonly diagnosed, causing the highest mortality rates. It was determined that lung cancer resulted from multiple somatic genetic changes in the critical genes (tumor suppressor gene and growth-promoting oncogenes) controlling cell proliferation, differentiation, and apoptosis (Sozzi, 2001). Lung cancer is divided into two types as small cell (SCLC) and non-small cell lung cancer (NSCLC) in terms of its histological differences, treatment modality, and prognosis (Damadoğlu, 2007). Chemotherapy has long been an effective treatment modality for lung cancer, and it remains one of the most critical elements of treatment for many patients. (Zappa and Mousa, 2016).

Pemetrexed (PEM) is one of the most effective cytotoxic agents used for NSCLC. PEM is a polyelectrolyte with two carboxyl groups containing guanidine N-1 in the pterin ring (Chattopadhyay et al., 2007). Unlike the conventional antimetabolite drugs like methotrexate that selectively targets one single enzyme, pemetrexed shows its activity by cutting several folate-dependent metabolic processes that are needed for cell replication through inhibiting the thymidylate synthase, glycinamide ribonucleotide formyl transferase, -and in more negligible amounts- 5-aminoimidazol 4-carboxamide ribonucleotide formyl transferase, dihydrofolate reductase enzymes. Since it targets many enzymes in folate metabolism, it is also called Multi-Targeted Antifolate (Rollins and Lindley, 2005). Drug delivery in cancer is essential for optimizing the effect of drugs and reducing toxic side effects. The use of nanoparticles in drug delivery facilitates drug delivery to tumors and overcomes most problems in traditional drug delivery (Singh and Lillard, 2009). Although conventional drug carriers only present drug molecules to the general circulation, nanoparticles can be adjusted in terms of half-life in the circulation with the help of various surface modifications, hiding from the immune system, having fluorescent or magnetic notifiers together with them, the carrier being programmed to deliver the drug into the cell, and when requested, the possibility of programming the DNA vectors that have the same specificity to be moved (Kocaefe, 2007).

Alginate is mucoadhesive, biocompatible, and non-immunogenic an anionic polymer produced by brown algae and bacteria, and consists of α-L-guluronic acid (G) and β-D-mannuronic acid (M) residues, linearly linked by 1,4-glycosidic linkages. Chitosan (CS) is a natural polysaccharide composed of the deacetylation of chitin and is constituted of a repeated unit of β-(1-4)-D-glucosamine units and β-(1-4)-N-acetyl-glucosamine. Alginate and chitosan polymers are non-toxic, biodegradable, and biocompatible substances. In addition, they are also advantageous for creating drug delivery systems because they facilitate chemical modification, have low cost, and are readily available (Lizardi-Mendoza et al., 2016; Paques et al., 2014).

Here, we reported a new drug delivery system for PEM based on alginate and chitosan used as nanocarriers to enhance their targeting. Alginate/chitosan nanoparticles (ACNP) have been

classically used as carrier systems for classical chemotherapeutic drugs; however, in our work, they were used as carriers for new-generation antifolate drug, PEM. In this work, we developed pemetrexed-loaded alginate/chitosan nanoparticles (PACNP) for non-small cell lung cancer therapy. ACNP were synthesized with the ionotropic gelation method. Calcium ions induced the gelation of alginate during the synthesis stage, and the resulting gel structure was covered with chitosan, which is a polycationic polymer. Pemetrexed was loaded on ACNP by the adsorption technique and nanoparticles were characterized.

2. Material and Method

2.1. Material

In this study, pemetrexed disodium was purchased from AK Scientific, chitosan (deacetylation degree ≥90%) was supplied from Marine Bio Resources, alginate was obtained from Sigma-Aldrich. Sodium hydroxide (NaOH) and sodium phosphate monobasic were supplied from Riedel-de Haen, calcium chloride ($CaCl_2$) was obtained from JT Baker, hydrochloric acid (HCl), and acetic acid (80%) were purchased from Tekkim, Sodium acetate anhydride was supplied from Merck. Dialysis tubing membrane (MWCO: 12000-14000 Da) was purchased from Sigma-Aldrich. All other chemicals were of analytical grade and were used as received.

2.2. Methods

2.2.1. Synthesis and characterization of nanoparticles

In the study, ACNP were synthesized using the method described by Ak et al. (2021). During the synthesis stage, the gelation of alginate was induced with calcium ions, and the resulting gel structure was covered with chitosan, which is a polycationic polymer. 18 mM CaCl2, 0.06% (w/v) alginate (in water), and 0.05% (w/v) chitosan (in acetic acid) solutions were prepared. pH of the alginate solution was adjusted to 4.9 and that of the chitosan solution to 4.6. $CaCl_2$ solution was added dropwise to the alginate solution during mixing at 22000 rpm using a homogenizer. Chitosan solution was added into the gel phase and incubated. Then the suspension was centrifuged. Resulting nanoparticles were collected, washed, and characterized with Fourier Transform Infrared Spectroscopy (FTIR) (IRTracer-100, Shimadzu) in Ege University Drug Development and Pharmacokinetic Research - Application Center (ARGEFAR) and Scanning Electron Microscope (SEM) (Thermo Scientific Apreo S) analyses in Ege University Central Research Test and Analysis Laboratory Application and Research Center (MATAL).

2.2.2. Adsorption of pemetrexed on ACNP

Pemetrexed was loaded on the ACNP through the adsorption technique. Firstly, varying concentrations of pemetrexed (0.5, 1, 1.5, 2, 2.5 and 3 mg/mL) solutions were prepared. Then, 1 mL pemetrexed solution was added into the ACNP. The mixture was incubated at 250 rpm at 25°C for 16 h in the orbital mixer (IKA, KS 130 basic). At the end of the period, the mixture was centrifuged at 13000 rpm, and the non-adsorbed drug was removed from

nanoparticles. ACNP were also washed twice with d-water (Hamarat Sanlier et al., 2016). The supernatant was used to analyze the non-adsorbed drug, and spectrophotometric determinations were performed at 227 nm using UV-Visible Spectrophotometer (Perkin Elmer Lambda 35). Adsorbed drug amount Eq.(1) and adsorption yield Eq.(2) were calculated according to the following equations:

Adsorbed drug amount (μg drug/mg nanoparticle)= (Initial drug amount (μg)-Nonadsorbed drug amount(μg))/(Nanoparticles' weight (mg)) **(1)**

Adsorption efficiency (%)= (Initial drug amount (μg)– Nonadsorbed drug amount (μg))/(Initial drug amount (μg)) x 100 **(2)**

After drug loading tests, PACNP were also investigated with FTIR and SEM for chemical and morphological examinations.

2.2.3. In vitro drug release

In vitro drug release studies were performed using the method Arias et al. (2012) described. To identify drug release from nanoparticles, PACNP containing 1 mg PEM were placed in a dialysis tubing membrane and dialyzed against 10 mL of PBS buffer (pH 7.4) and acetate buffer (pH 5.5) for 25 h at 37°C, under conditions of constant shaking. Dialysis buffers were changed with fresh buffer at specific periods. The free pemetrexed was also used for release analysis under the same conditions. The amount of released pemetrexed was spectrophotometrically determined from the receiver solution. The cumulative release was calculated with Eq. (3) and compared with free drug release.

Cumulative release (%)= (Amount of released PEM (μg))/(Initial amount of PEM (μg)) ×100 **(3)**

3. Results and Discussion

3.1. Synthesis and characterization of nanoparticles

ACNP were synthesized according to the ionotropic gelation method and characterized with FTIR and SEM to determine chemical structure, morphology, and size. It was ensured that the Ca^{2+} ions induced alginate to form an egg-box-like structure, and the pre-gel were coated with chitosan, a cationic polymer, for stabilization.

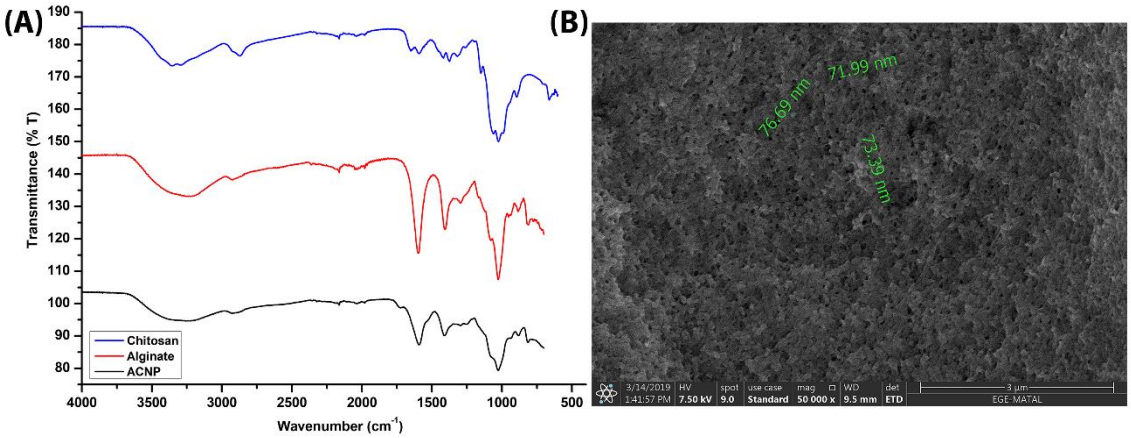

Figure 1. FTIR spectra of (A) Chitosan, Alginate, ACNP and (B) SEM images of ACNP.

In FTIR spectrum of CS (Figure 1(A)), the prominent peak formation was detected at 3500-3000 cm^{-1}, corresponding to the amine and hydroxyl groups. The peak at 2875 cm^{-1} was caused by -OH stretching, and the absorption band of the carbonyl stretching of the secondary amide was observed at 1643 cm^{-1}, and the bending vibrations of the N-H were determined at 1597 cm^{-1} (Ak et al., 2020; Kaya et al., 2014). The bands around 1024 cm^{-1} (C-O-C stretching) presenting in the FTIR spectrum of sodium alginate (Figure 1(A)) were attributed to its saccharide structure. Also, the bands at 1602 and 1400 cm^{-1} were appointed to asymmetric and symmetric stretching peaks of carboxylate salt groups (Daemi and Barikani, 2012).

After the complexation of chitosan with alginate (Figure 1(A)), the stretching vibration of O-H and N-H at 3500-3000 cm^{-1} in CS became broader. However, both peaks were shifted after complexation. The amino peak shifted from 1597 to 1594 cm^{-1} and became stronger. The carboxyl peak of sodium alginate shifted from 1400 cm^{-1} to 1410 cm^{-1}. The bands around 1024 cm^{-1} (C-O-C stretching) shifted slightly from 1024 cm^{-1} to 1025 cm^{-1} and became broader (Gazori et al., 2009; Ji et al., 2019). The differences between FTIR spectra of alginate, chitosan, and alginate/chitosan nanoparticles displayed the interactions of alginate and chitosan.

According to the SEM images (Figure 1(B)), ACNP had spherical morphology, and the size of ACNP was approximately 75 nm like as in a study conducted by Zhang et al. (2016). Nanoparticles, which are about 100 nm in size, are suitable for passive targeting and drug delivery, so that ACNP were investigated for drug loading in the following studies.

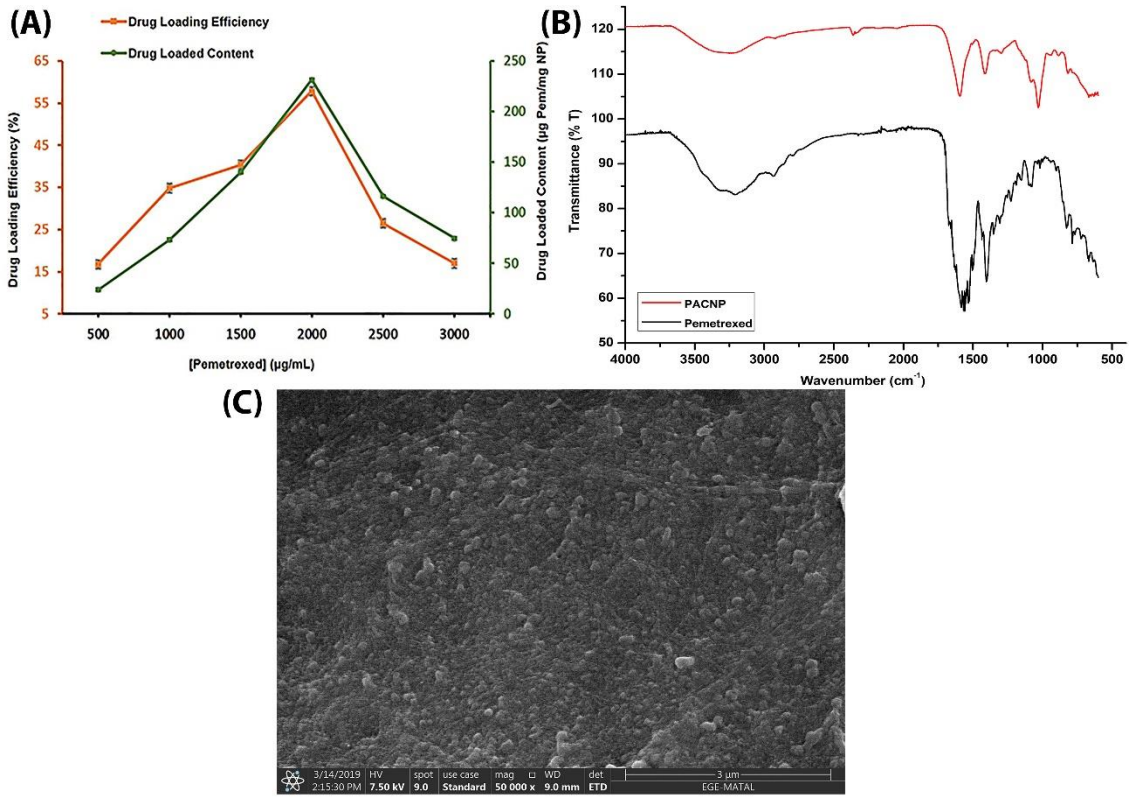

Figure 2. (A) Drug loading efficiency (%) and amount (µg PEM/mg nanoparticle) of pemetrexed on ACNP. (B) FTIR spectra of PACNP, Pemetrexed (C) SEM images of PACNP.

3.2. Adsorption of pemetrexed on ACNP

The loading of PEM on ACNP was achieved utilizing the adsorption method. As seen in Figure 2(A), different concentrations of PEM were loaded on ACNP. When the adsorption yields of the drug-loaded nanoparticles were examined, it was determined that the adsorption yield increased gradually in initial increasing drug concentrations, reached a maximum level at 2 mg/mL drug concentration, and the adsorption yield decreased when this concentration was exceeded. When 2 mg/mL initial drug concentration was used, the loaded drug amount was determined as 230 µg drug/mg nanoparticle, and the drug loading yield was found to be 57.80%. Similarly, Chen et al. (2014) synthesized chitosan nanoparticles loaded with methotrexate, and the drug loading efficiency was found to be 44.19%. The optimum initial PEM concentration was chosen as 2 mg/mL since it was thought that excessive drug molecules obstacle drug interactions with nanoparticles.

The characteristic peaks of PEM Figure 2(B) showed broadband shaped peak at 3100–3400 cm^{-1} belong to O–H and primary amine (N–H) groups, the peak at 2930 cm^{-1} belongs to C–H stretching, the signal at 1404 cm^{-1} belongs O–H bending of carboxylic acid and the peak at 1086 cm^{-1} belongs to C–H bending in the FTIR spectrum (Ak et al., 2019). In the FTIR spectrum of PACNP (Figure 2(B)), there was an increase in the density of the bonds at 1404 and 1594 cm^{-1} showing an increased signal of O–H bending of carboxylic acid and amine groups. This is due to the inclusion of pemetrexed in the ACNP structure. Because of the

presence of alginate and chitosan polymer, some additional peaks were present.(Kumari et al., 2013).

As a result of the SEM analysis of the drug-loaded nanoparticles, it was determined that there were no changes in the size of the nanoparticles (Figure 2(C)). Nanoparticles were still spherical and approximately 80 nm. Ak and coworkers developed pemetrexed loaded magnetic O-carboxymethyl chitosan nanoparticles (PMCMC) for pemetrexed delivery. The hydrodynamic size was detected as 123.9 nm and nearly identical with empty nanoparticles. In addition, the spherical structure remained upon SEM examination. No significant size change was observed in both studies after pemetrexed loading (Ak et al., 2019).

Size is an important feature of nanoparticles that plays key role in their internalization by cells. It has been known that nanoparticles between 15 and 100 nm are ideal for long-term circulation. A study also claimed that nanoparticles herein also displayed a size below 100 nm, making them ideal for delivery of drugs to the cells involved in the lung tumor (Bakhtiary et al., 2017; Chiarelli et al., 2015).

3.3. In vitro drug release

Drug release profiles of PEM and PACNP were examined at both acidic medium (pH 5.5) and physiological medium (pH 7.4) for 25 hours. Figure 3(A) shows the release profile of free drug. The release of free drug reached the ratios of 50% at pH 5.5 and 25% at pH 7.4 in the first 5 h. The release was about two times slower in the physiological medium (pH 7.4) than in the acidic medium (pH 5.5). Figure 3(B) demonstrates the release profile of PEM from nanoparticles. In pH 5.5 medium, drug release from nanoparticles was found as 4, 8, and 11% at the 1st, 4th, and 25th hours, in order. While in pH 7.4 medium, the release of drug from nanoparticles was found as 2, 5, and 7% at the same time intervals, in turn Soni and coworkers prepared lipid drug conjugated nanoparticles for pemetrexed delivery by using stearic acid. The release ratio of pemetrexed from nanoparticles was 83.56% after 25h, compared with the release ratio of pure pemetrexed 94.85% at the end of 4 h (Soni et al., 2017).

In order to understand the drug release mechanisms, after in vitro release studies, the results were fitted in various kinetic models. The best suitable model was decided by correlation coefficient (R^2) value. The release of pemetrexed from ACNP in pH 5.5 was in accordance with the Higuchi ($R^2 = 0.9908$) kinetics (Figure 3(C)). This model has based on the hypothesis that drug diffusion occurs only in one dimension and that swelling of matrix and dissolution is considered to be less or insignificant while maintaining sink condition. (Affram et al., 2020; Farshi Azhar and Olad, 2014). Küçüktürkmen and coworkers developed lipid-polymer hybrid nanoparticles for pemetrexed and miR-21 antisense oligonucleotide delivery. It was aimed to treat glioblastoma, the most aggressive type of brain tumor. Encapsulation of pemetrexed in nanoparticles increased cellular uptake. Nanoparticles had Higuchi and Korsmeyer–Peppas release kinetics. Consistency with Higuchi kinetics showed that pemetrexed, which is a hydrophilic drug, is primarily released by diffusion (Küçüktürkmen et al., 2017).

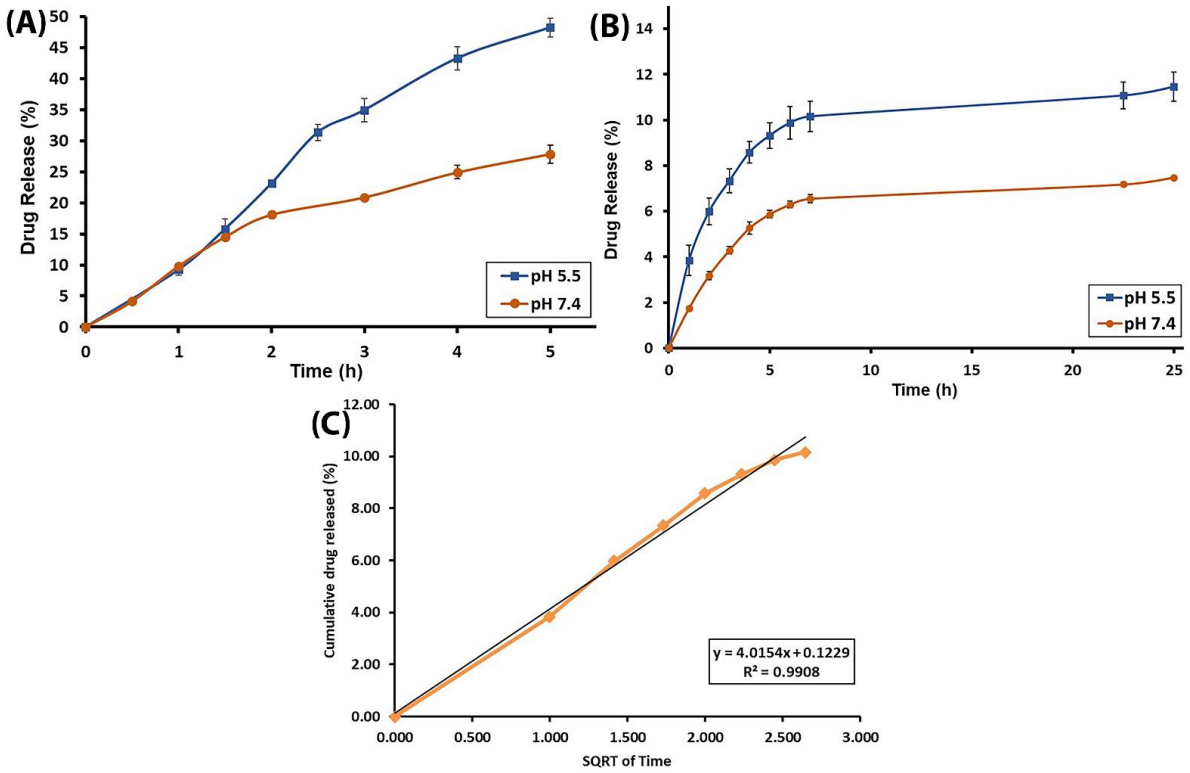

Figure 3. Cumulative drug release (%) of pemetrexed (A) from free form and (B) from ACNP in different pH media (pH 5.5 and 7.4) (C) Higuchi kinetic model of pemetrexed release from PACNP.

According to all these data, it was determined that the release of drug from nanoparticles was higher and occurred in prolonged periods in acidic conditions than in physiological conditions compared to free drug. For this reason, PACNP could be more toxic to lung cancer cells (due to the acidic microenvironment) than healthy cells was concluded that nanoparticles had controlled release characteristics, and the side effects of pemetrexed could be minimized by using PACNP.

4. Conclusion

It has been known that pemetrexed has antitumor activity on non-small cell lung cancer. In this study, we have synthesized pemetrexed loaded alginate/chitosan nanoparticles for delivery of pemetrexed for lung cancer therapy. ACNP were synthesized with the ionotropic gelation method. Pemetrexed was loaded on ACNP via the adsorption technique. According to the data in the literature, particles below 100 nm are suitable for passive tumor targeting with increased permeability and retention effect. As a result of, PACNP have 80 nm in size, and PACNP have a controlled drug release profile so that it could be used for passive tumor targeting. In this study, it was observed that drug release was higher in acidic conditions compared to physiological conditions, and controlled prolonged-release occurred from nanoparticles compared to free drug. Based on data in the literature, the pH level in the tumor's microenvironment decreases to a range of 5 – 6.5. The analyses were performed at pH

7.4, which is similar to physiological conditions, and at pH 5.5, which is similar to the acidic medium of the tumor cell. This pH-sensitive release of PACNP could effectively enhance the cell internalization of drugs and inhibit lung cancer cell proliferation. This pH-dependent pemetrexed release property of PACNP is beneficial for drug delivery systems in lung cancer therapy because most lung tumors present more acidic conditions than normal tissues. Thus, PACNP could have the potential in drug delivery and releasing therapeutics for treating lung cancers. Further studies should include studies on cell culture and in vivo animal studies to determine the biodistribution profile of nanoparticles.

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