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Composite Cryogels for Drug Delivery Applications: A Preliminary Study with Dye as a Model Drug

Didem Demir[1], Seda Ceylan[2], Nimet Bölgen[3]*

[1]Chemistry and Chemical Process Technologies Department, Mersin Tarsus Organized Industrial Zone Technical Sciences Vocational School, Tarsus University, Mersin, 33100, Türkiye

[2]Bioengineering Department, Faculty of Engineering, Adana Alpaslan Türkeş Science and Technology University, Adana, 01250, Türkiye

[3]Chemical Engineering Department, Faculty of Engineering, Mersin University, Mersin, 33110, Türkiye

Abstract: Cryogels are suitable candidates to be used as drug release systems due to their interconnected pore structures, high surface areas, high liquid absorption capacities, and elasticity. With this purpose, we aimed to produce a cryogel structure to be used in drug release applications with the approach of tissue engineering. As biodegradable and biocompatible polymers chitosan and gelation were selected. The cryogels were fabricated using the combination of these polymers in the presence of glutaraldehyde under cryogenic conditions. The produced optimum gel scaffold was first characterized using FTIR, SEM, porosity, swelling ability, and degradation analyses. Successfully crosslinked gels exhibited an interconnected pore structure with an average pore diameter of 52.95 µm. As a result of the examination of the time-dependent weight change, it was also revealed that the cryogels have a liquid absorption capacity of about 500 times their dry weight and are biodegradable. The mainly characterized cryogel sample was evaluated for potential drug loading and release applications using methyl orange (MO) as a model drug. Gels, which swell in a short time, absorb the dye quickly and the cumulative release of the dye indicates that the gels are suitable for extended-release systems.

Keywords: Chitosan, gelatin, cryogel, drug delivery.

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***Corresponding author. E-mail:** nimetbolgen@yahoo.com.

1. INTRODUCTION

The main goal of tissue engineering is restoring, repairing, and maintaining damaged tissue functions with cells, scaffolds, and growth factors (Biondi et al., 2008). Recently, incorporating different drugs (drug active ingredients, small molecule chemicals, proteins, growth factors, cytokines, and other bioactive molecules) into tissue engineering scaffolds has gained a lot of attention, and has great potential in biomedical applications (Mondal et al., 2016). Drug delivery or device system provides optimum dose control on the specific targeted tissues and also decreases the side effects of drugs on the non-targeted tissues as compared to the traditional drug delivery treatments (Yusop et al., 2018). In other words, the selectivity ability of drugs in treating targeted cells for disease treatment is important to protect healthy parts of the body (Surya et al., 2020). In light of this knowledge, the porous structure of the scaffolds has been loaded with various drug types such as painkillers, anti-inflammatory, antimigraine and anti-cancer agents and hormones (Piazzini et al., 2019). Hydrophobic and hydrophilic model drugs (PKH26 or PKH67, methyl orange, methylene blue, etc.) have also been loaded into scaffolds (Kim et al., 2005). In

recent years, using natural or synthetic polymers has attracted wide attention in producing different types of scaffolds and combining them with drugs. Various scaffold production techniques, such as gas foaming, electrospinning, thermal phase separation, emulsification, solvent casting, freeze drying, cryogelation, etc., are used to produce scaffolds. Natural (silk, chitosan, gelatin, starch, pectin, cellulose, etc.) and synthetic polymers (poly (vinyl alcohol) (PVA), poly (lactic-co-glycolic acid) (PLGA), poly-L-lactide (PLA), etc.) based scaffolds obtained by using different techniques have been reported for controlled drug delivery applications in various studies (Shera et al., 2018; Wang et al., 2019). Curcumin-loaded PLGA particles (Yusop et al., 2018), vitamin B12-loaded alginate scaffolds (Bhasarkar & Bal, 2019), recombinant human bone morphogenetic protein 2 and dexamethasone-loaded silk fibroin/PLGA scaffolds (Yao et al., 2019), 5-fluorouracil-loaded nanocellulose/gelatin cryogels (Li et al., 2019) and doxorubicin containing chitosan hydrogel (Han et al., 2008) are impressive examples produced in different researches with updated strategies to design biomimetic scaffolds.

In this study, chitosan and gelatin natural polymers and cryogelation technique were used to produce porous cryogel scaffolds for potential drug loading and release experiments. Chitosan and gelatin are widely used natural polymers due to their favorable properties such as biological compatibility, biodegradability, non-toxicity, and high water absorption capacity (Ayaz et al., 2021). On the other hand, cryogelation is a favorable method to produce three-dimensional structures with highly interconnected porosity (Rogers & Bencherif, 2019). The excellent features of chitosan and gelatin and the use of cryogelation technique are notable for accepting the fabricated scaffolds as excellent candidates for biomedical applications. Up to now, in different research studies, chitosan and gelatin have been incorporated as implantable scaffolds such as wound dressing, bone tissue engineering, neural regeneration, and vehicles for controlled delivery of therapeutic molecules (drug, protein, gene, etc.) (Bhat et al., 2011; Kemençe & Bölgen, 2017, 2017; Lu et al., 2004; Nagahama et al., 2009). Our study aims to reveal the drug loading and release potential of scaffolds prepared in a combination of chitosan and gelatin. For this, first of all, plain chitosan, plain gelatin, and half by weight of chitosan and gelatin cryogels were prepared. After the scaffolds were evaluated structurally, characterization studies were carried out with composite scaffolds. The cryogels were analyzed by using chemical composition, morphology, swelling ability, and degradation behavior experiments. Then, preliminary studies on drug loading and drug release experiments were carried out by using methyl orange as a model drug. The results obtained from the study will contribute to the estimation of real drug release profiles, determine the basic physicochemical properties of a biocompatible and biodegradable material that can release drugs, and shed light on the future biological studies of the material.

2. EXPERIMENTAL SECTION

2.1. Materials

Chitosan with low molecular weight was purchased from Sigma Aldrich, USA. Gelatin (for microbiology), glutaraldehyde (25%, v/v), glacial acetic acid (100%, v/v), and methyl orange azo dye were received from Merck, Germany. Aqueous solutions and dilutions in experiments were prepared with distilled water.

2.2. Preparation of Gelatin: Chitosan Composite Cryogels

The solvent of the polymers was selected according to our previous studies as acetic acid with a volumetric ratio of 6%. To prepare the polymer solution, chitosan and gelatin were dissolved separately in an acetic acid solution and distilled water, respectively. Chitosan solution (2%, v/v) was prepared by dissolving the calculated amount of chitosan in 20 mL of 6% acetic acid solution by stirring for 24 hours. Also, the pre-determined amount of gelatin was dissolved in 20 mL of distilled water and stirred for 24 hours. Homogeneous gelatin: chitosan solution was obtained by thoroughly mixing the prepared chitosan and gelatin solutions at different volumetric ratios (100:0, 50:50, and 0:100, gelatin: chitosan). 0.5 mL of glutaraldehyde solution was added immediately to 2 mL of polymer solution and the as-prepared solution was loaded into a 2.5 plastic syringe to give cryogel a monolithic shape. The syringe was rapidly placed in cryostat and incubated at -16 °C for 3 h. At the end of the incubation time, the sample was stored for 24 h in the freezer at -16 °C. The prepared samples were thawed at room temperature and washed repeatedly with distilled water to remove the unreacted ingredients. The samples were then freeze-dried. The dried samples were stored in the refrigerator at +4 °C for further analysis.

2.3. Characterization Studies of Cryogels

Different physical, chemical, and morphological analyses were used to characterize the fabricated cryogels for further usage in drug loading and drug release studies.

Main chemical groups and, interactions between polymers and crosslinking agents were determined using Fourier Transform Infrared spectroscopy (FTIR) (PerkinElmer, FTIR/FIR/NIR Spectrometer Frontier-ATR, USA). The infrared spectra of the scaffold and polymers were measured in the wavenumber range of 4000-450 cm^{-1}.

The morphology and microstructure of cryogel were investigated using scanning electron microscopy (SEM, FE-SEM Zeiss/Supra55, Quanta 400F Field Emission, USA). The cryogel samples were prepared by coating them with a thin layer of platinum before analysis. SEM was operated at the acceleration of 5 kV and the magnification was

200x. The average pore diameter was calculated by measuring at least 50 pores of cryogel using Image-J software.

The water absorption capacity of the completely dried cryogels was determined using the gravimetric method. Dry cryogels were weighed and (a cylindrical shape with 9 mm diameter and 5 mm height, n=3) were immersed in PBS at 37 °C. At certain time intervals, the swollen cryogels were gently transferred to a filter paper to remove the excess water from the surface and weighed again. The swelling ratio of the samples was calculated according to Eq. 1;

$$SR(\%)=\left[\frac{(M_f-M_i)}{M_i}\right]\times 100 \quad \text{(Eq. 1)}$$

where Mi is the initial dry weight of the scaffold, Mf is the swollen weight of the scaffold and SR is the swelling ratio (Demir et al., 2020).

The cryogels were cut into cylindrical pieces (radius= 9 mm, height= 3 mm, n=3) and the dry weight of cryogels was recorded. After that, the samples were incubated in 20 mL centrifuge tubes filled with sterile PBS solution at 37 °C in a shaking water bath (Daihan Scientific Co. Ltd., WiseBath WB-22, Korea). At pre-determined time intervals, samples were washed with distilled water and freeze-dried. After the drying process, the weight of each sample was recorded and the weight loss percentage was calculated according to the Eq. 2;

$$DD(\%)=\left[\frac{(W_i-W_f)}{W_i}\right]\times 100 \quad \text{(Eq. 2)}$$

where Wi is the initial dry weight of the scaffold, Wf is the final dry weight of the scaffold, and DD is the degree of degradation or degradation rate (Demir et al., 2021).

2.4. Model Studies with Dye for Drug Loading and Drug Release

MO was used as the model drug to investigate the drug loading and drug release potential of the fabricated Gel:Cs cryogel scaffold, similar to the use of different dyes as model drugs in other studies previously (Demir et al., 2018; Hauck et al., 2022; Khansari et al., 2013; Pancholi et al., 2009). For the loading of the dye, the cryogel sample was immersed in 5 mL of MO solution (0.05 mg/L) at room temperature. After 48 h incubation, samples were removed from the dye solution and rinsed with a known volume of pure water. The weight of dye was analyzed by UV-Vis spectroscopy (Chebios Optimum-One UV-vis, Italy) at a wavelength of 554 nm. The dye encapsulation efficiency of each sample was calculated using Eq. 3.

$$EE,\%wt=\left[\frac{C_{dye,i}-C_{dye,w}}{C_{dye,i}}\right]\times 100 \quad \text{(Eq. 3)}$$

where EE is the encapsulation efficiency, $C_{dye,\ I}$ is the concentration of dye in the initial solution, and $C_{dye,w}$ is the concentration of dye in the washing solution.

The release of MO from the cryogels was evaluated in phosphate buffer saline (PBS at pH 7.4) solution. MO-loaded samples were immersed in plastic tubes filled with 10 mL of release medium. The tubes were incubated in a shaking water bath at 37 °C with 100 rpm. 3 mL of release medium were withdrawn at pre-determined time intervals (5, 15, 30, 60, and 240 min) and replaced with an equal volume of fresh medium. Weight of MO release was quantified by UV-Vis spectroscopy. The percentage release of the MO was calculated as cumulative using Eq.4.

Cumulative Dye Release, %wt= [(Weight of dye released)/(Weight of dye in the cryogel)]*100 (4)
The dye absorption and release experiments were done in triplicate and results are presented as mean ± SD.

3. RESULTS AND DISCUSSION

3.1. Cryogel Synthesis and Characterization

The experimental setup, cryogelation process, and typical crosslinking between gelatin and chitosan with glutaraldehyde are summarized in Figure 1. Cryogelation technique, in other words, crytropic gelation, was used in the production of scaffolds to create an interconnected macroporous structure for more effective drug absorption and release studies. In the cryogenic process, gelation occurs at subzero temperatures, leading to the formation of a polymeric network crosslinked around the icy crystals. Then, with the thawing of these crystals, an interconnected macroporous structure surrounded by highly dense polymeric walls remains. In this study, we used gelatin and chitosan as naturally derived polymers to fabricate cryogels. A general overview of the experimental setup, crosslinking mechanism, and cryogelation technique was shown in Figure 1.

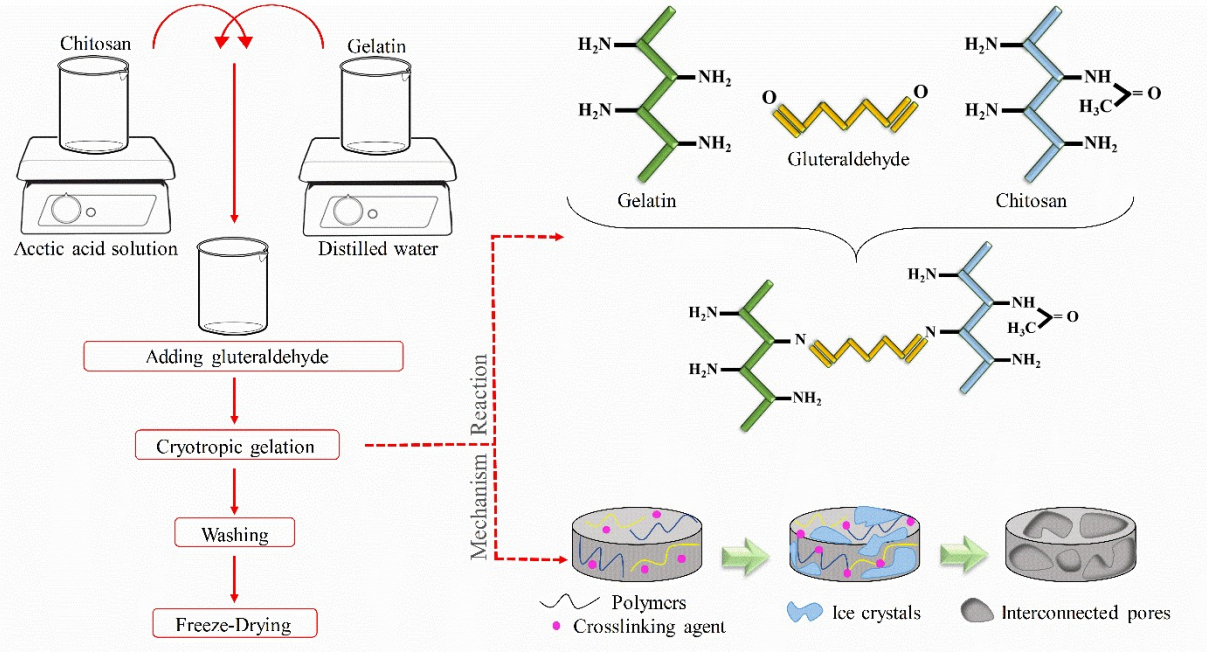

Figure 1: General overview of the experimental set-up, crosslinking mechanism, and cryogelation technique.

Before using chitosan and gelatin together, we wanted to observe the potential for scaffolding by using chitosan and gelatin separately. As the structural properties are shown in Table 1, gelatin alone exhibited a looser structure, while chitosan exhibited a more brittle and hard structure. When both polymers are used alone, materials that are not easy to apply and that are not stable enough have been produced. The composite produced with the use of chitosan and gelatin by half by weight, on the other hand, exhibited a stable, water-capable structure that could return to its original state after releasing the absorbed water. For this reason, in the continuation of the study, the composite scaffold was selected as the optimum sample.

Table 1: Effect of chitosan: gelatin ratio on the production of cryogel (Other parameters are the same).

Chitosan:Gelatin Ratio	Morphology
100:0	Fragile
50:50	Spongy
0:100	Bursting with high swelling

The chemical bond structure of the polymers used and the changes in their structures as a result of crosslinking with glutaraldehyde were analyzed with the FTIR spectra presented in Figure 2. Both polymers exhibited general characteristic bond structures before processing. For chitosan, the absorption bands around 2977 and 2888 cm^{-1} are related to C-H symmetric and asymmetric stretching, respectively. The peaks found at 1645, 1550, and 1380 cm^{-1} are attributed to Amide I (C=O stretching), Amide II (N-H bending), and Amide III (C-N stretching), respectively (Demir et al., 2016; Fernandes Queiroz et al., 2014). For gelatin, there are four major peaks located at 3230, 1631, 1524-1315, and 1240-698 cm^{-1} which correspond to Amide A, Amide I, Amide II, and Amide III regions, respectively (Pradini et al., 2018). After crosslinking the polymers in the presence of glutaraldehyde, it was observed that the intensity and positions of the peaks changed significantly. The absorption peak of the free amino group (Amide A) and OH group shifted from 3230 to 3300 cm^{-1}. The spectrum showed four strong absorption peaks at 1640, 1555, 1405, and 1068 cm^{-1} which were formed by the overlapping of the chitosan and gelatin peaks. In addition, a new absorption peak was formed at 1030 cm^{-1} which was attributed to C–O–C–O–C structure after cross-linking (Qian et al., 2011).

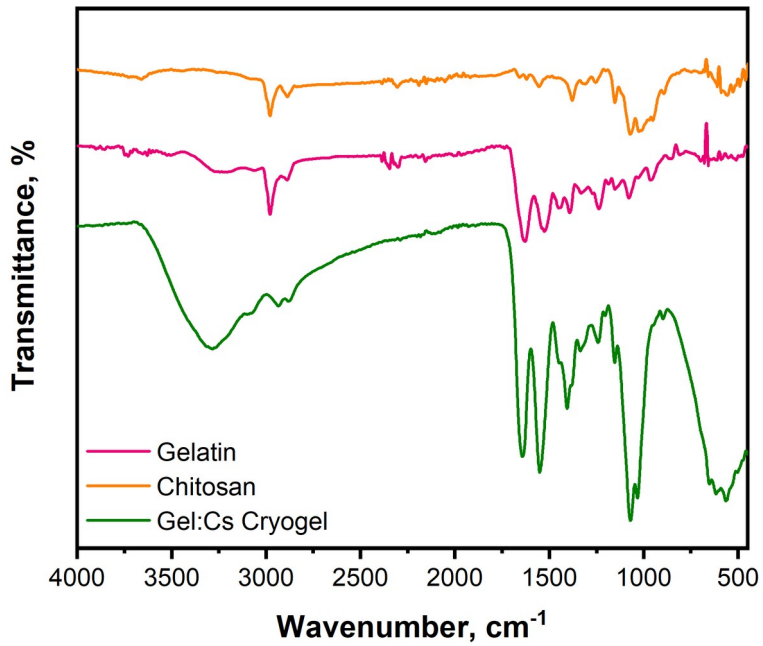

Figure 2: FTIR spectra of gelatin, chitosan, and crosslinked Gel:Cs cryogels.

To investigate the morphology of crosslinked Gel:Cs cryogel SEM images were obtained as seen in Figure 3A. The scaffold showed an interconnected porous structure as a result of the cryogelation process. In the SEM image, the gray parts represent the crosslinked polymer walls, while the dark parts show the pores. The porous structure of the material is important for cell adhesion, diffusion, migration, and proliferation in terms of tissue regeneration/new tissue formation, while it is important for drug release studies in terms of providing high drug absorption capacity and drug diffusion. In this context, it is also necessary to determine the diameter of pores. Therefore, the diameter of at least 50 pores was measured and a histogram was obtained as seen in Figure 3B. It is seen that the pore diameter range varies between 27.89 and 97.49 µm. The mean diameter was calculated at 52.95 µm. When other studies are examined, these values vary between 10-50 µm for silk cryogels (Ak et al., 2013), 15-45 µm for chitosan pectin cryogels (Demir et al., 2021) and 30-100 µm for pHEMA -poly(ethylene glycol) diacrylate-gelatin cryogels (Singh et al., 2011). In addition, the porosity of cryogel was examined gravimetrically using the ethanolic penetration method and was found as 57.05%.

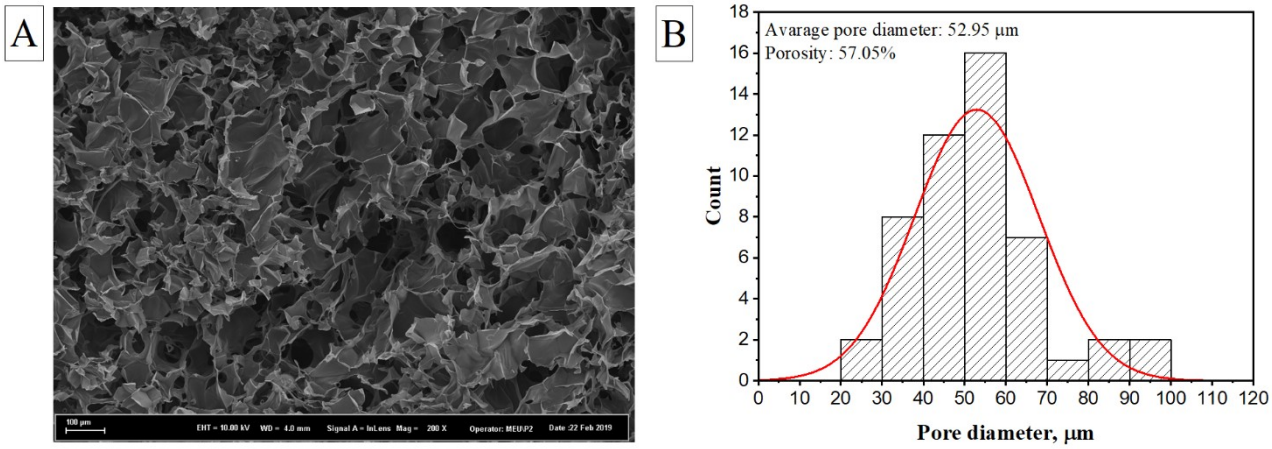

Figure 3: A) SEM image showing the porous structure of the Gel:Cs cryogels and B) Pore diameter histogram of Gel:Cs cryogel

According to the porous structure of the scaffold and the hydrophilic structure of the polymers, it was estimated that the gels can absorb high amounts of liquids. For this reason, the swelling ratios and equilibrium swelling ratios were calculated by gravimetrically measuring the change in weight of the completely dry samples after they were kept in PBS for certain periods (5,

15, 30, 60, 90, 120, 150, and 180 min). In Figure 4A, it is seen that the cryogel starts to absorb water within the first 5 minutes and reaches the equilibrium point at the end of 180 minutes. The high liquid absorption ability of the gels (approximately 500 times its own dry weight) is an important feature shows that the drug solutions can be absorbed easily into the structure of the gel scaffold. This hydrophilicity is due to the presence of carboxyl, amino, and hydroxyl groups in the polymer backbone as well as the highly porous structure of the sample (Hezaveh et al., 2012; Vo et al., 2021). When other studies are examined, it is seen that the swelling ratio values are quite high, especially for cryogels with highly interconnected macroporosity (Meena et al., 2018).

The degradation behavior of the cryogel was studied to determine whether it affects its stability and drug release behavior. For this purpose, dry gels were taken into PBS and the change in their weight was recorded for 4 weeks, and their degradation rates were monitored (Figure 4B). The sample began to degrade by 6.68±0.61% of its initial weight within the first week. Afterward, it lost 20.95±1.81% of its initial weight in 1 month. Degradation of polymeric biomaterials occurs as a result of the breaking of hydrolytic or enzymatically sensitive bonds that cause polymeric erosion. In the meantime, molecules such as drugs loaded in the polymeric system can be released into the environment depending on the degradation rate (Ghanbarzadeh & Almasi, 2013). Therefore, the drug release rate varies depending on the degradation rate of the materials. For example, in a therapy where a long duration is needed, materials with slow degradation kinetics are needed (Martins et al., 2018). Here, chitosan and gelatin, as biodegradable natural polymers, appear to exhibit faster degradation behavior than synthetic polymers, suggesting that Gel:Cs cryogel can be used as drug delivery systems for therapies with shorter durations.

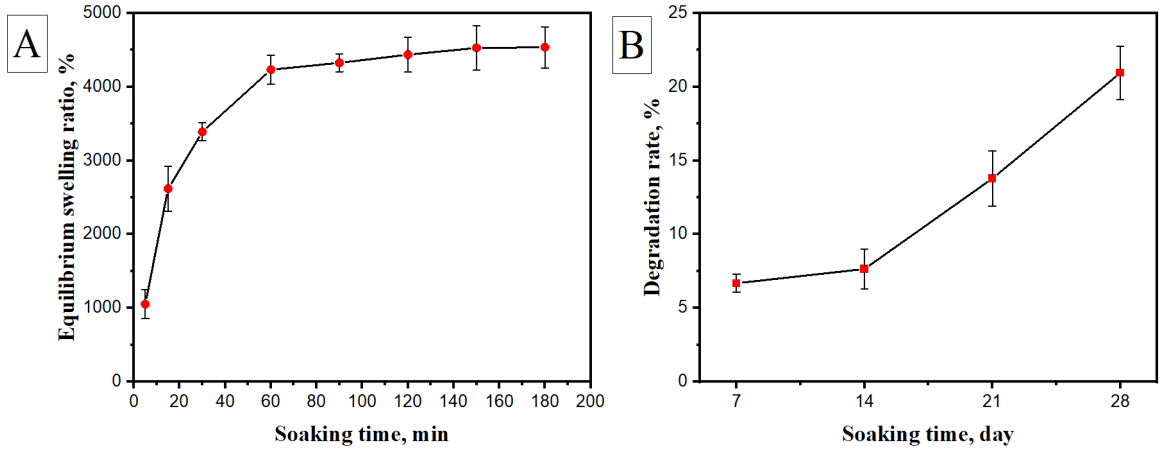

Figure 4: A) Swelling ratio versus soaking time of Gel:Cs cryogel in PBS pH 7.4 and B) Degradation rate of cryogel up to 28 days.

3.2. Dye Loading and Dye Release Behavior of Cryogels

After determining the main physicochemical properties of cryogel sample, dye loading and dye release studies were performed (Figures 5A and 4B). In these experiments, MO was used as a model drug. The cryogel samples in disc shapes were placed in an aqueous concentrated MO solution and allowed to equilibrate. It is seen that the cryogel discs absorb approximately 45% of the initial dyestuff amount after 120 minutes. In order to find the equilibrium value, the cryogels continued to hold in the dye solution and the amount of dye loaded at the end of 4 days was calculated as 50.45±4.02%. The color change in the cryogel due to the orange color of the MO before and after dye loading is also seen in the images embedded in Figure 5A. This situation can be explained by electrostatic interactions between active sites of adsorbent (Gel:Cs cryogel) and adsorbate (MO) molecules. The amine $-NH_2$ group in chitosan was able to accept a proton from a hydronium ion. As a result, electrostatic interaction easily occurred between NH_3^+ and MO^-; therefore, the cryogel showed good entrapment efficiency against MO (Loc et al., 2022). Based on the results, it can be said that Gel:Cs cryogels are suitable drug release systems for loading water-soluble, anionic, and weakly acid drug molecules such as MO.

To demonstrate the drug release behavior of MO-loaded gels, the cumulative release was monitored over time in a PBS buffer. The cumulative release of MO as a function of time is presented in Figure 5B. During the first 5 minutes of release, the cryogel provided about 3% of the MO release, while in the 90 minutes it released about 18%. The release rate reached 34.88±2.87% at the 30th minute and then slowed down and approached the equilibrium (32.94±4.01%) at the 180th minute. The rate of drug release from a polymeric system

can be affected by different events, from drug dissolution to water absorption, polymer swelling, drug dissolution through the polymer network, diffusion, and polymer erosion (Şarkaya & Allı, 2021). In our study, we can mainly relate the release during this 1 day to the rapid swelling properties of the cryogels. The high swelling capacity of the gels may have both accelerated the diffusion rate of the penetrant into the matrix and facilitated MO dissolution and diffusion of the swollen matrix through the gel layer, exhibiting a faster drug release profile up to the equilibrium swelling point of the cryogel (The cryogel reached equilibrium at a swelling rate of 4535.57±277.93% in 180 minutes, as presented in Figure 4A). The results obtained here are that a slow release occurs due to the swelling kinetics only, as a result of the strong electrostatic interactions between the MO and the polymers.

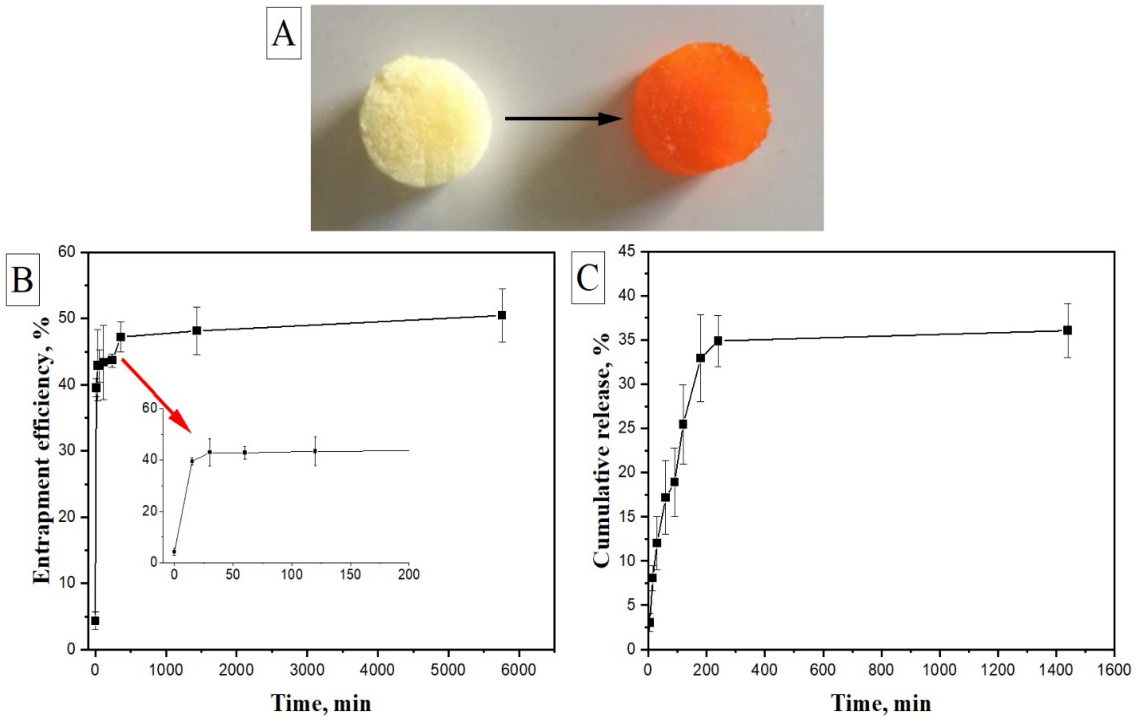

Figure 5. A) Color change in cryogel after dye adsorption, B) MO adsorption capacity versus time, and C) Time-dependent release of MO.

4. CONCLUSION

In this study, glutaraldehyde crosslinked cryogel scaffolds were fabricated, characterized, and then evaluated for drug adsorption and release studies. Considering that polymer type and the ratio is a critical production variables in cryogel production, plain chitosan, plain gelatin, and a composite scaffold combined half by weight of chitosan and gelatin were evaluated. It was observed that the composite scaffold exhibited mechanically a more stable and water-capable structure, and the study was continued with this sample. The composite cryogel showed an open network with an interconnected porous structure. The ability of cryogel as an adsorbent for MO from an aqueous dye solution was examined. In light of the experiments, the results indicate that glutaraldehyde crosslinked Gel:Cs cryogel has good characterization properties for drug loading and release applications. Using the appropriate formula and fabrication method can affect the cryogel scaffold stability and release MO, which can be targeted for drug or growth factor release for cell migration, proliferation, and formation of new tissue for future studies. This study demonstrates that we can optimize the formula of Gel:Cs cryogels and maximize the role of polymer ratio for further drug loading and release applications.

5. CONFLICT OF INTEREST

The authors have no conflict of interest.

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