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Research Article

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Preparation and Investigation of Antibacterial Activities of Ciprofloxacin Imprinted p(HEMAH) Cryogels

Siprofloksasin Baskılanmış p(HEMAH) Kriyojellerin Hazırlanması ve Antibakteriyel Aktivitelerinin Araştırılması

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ABSTRACT

Staphylococcus aureus, Enterococcus faecalis and Escherichia coli are the common causes of wound infections. For the treatment of these infections, ciprofloxacin can be recommended as a broad-spectrum antibiotic that acts on both Gram-negative and Gram-positive microorganisms. Besides, antimicrobial agents could be integrated into polymeric materials. Cryogels, one of these polymeric materials, are spongy polymers showing high macroporosity. In addition to their attractive usage as affinity support materials and scaffolds, they also appear as drug carrier materials in recent years. Molecular imprinting method is a recognition technique prepared by forming a polymeric network around the template. Although this method has been used in purification and separation processes for more than thirty years, it has gained great interest as a new approach that provides an advantage in drug release studies in terms of high drug loading capacity and long-term release. In this study, ciprofloxacin (CIP) imprinted 2-hydroxyethyl methacrylate (HEMA) based *N*-methacryloyl-(L)-histidine methyl ester (MAH) containing [CIP-*p*(HEMAH)] cryogels were prepared and characterized. CIP releasing experiments were performed, and then, antimicrobial activities of CIP *p*(HEMAH) cryogels were examined against *S. aureus, E. faecalis and E. coli*. It was found that cumulative CIP release ratios were indicated as 80.6% and 88.5% for CIP-*p*(HEMAH) cryogels at the end of 6 and 120 h, respectively. It can be concluded that CIP-*p*(HEMAH) cryogels could be proposed as promising polymeric materials for wound healing applications.

Key Words

Ciprofloxacin, molecular imprinting, cryogels, antimicrobial activity.

ÖZ

Staphylococcus aureus, Enterococcus faecalis and Escherichia coli yara enfeksiyonlarının yaygın nedenleridir. Siprofloksasin, bu enfeksiyonların tedavisi için hem Gram-negatif hem de Gram-pozitif mikroorganizmalara etki eden geniş spektrumlu bir antibiyotik olarak önerilebilir. Bunun yanı sıra, antimikrobiyal ajanlar polimerik malzemelere entegre edilebilirler. Bu polimerik malzemelerden biri olan kriyojeller, yüksek makro gözeneklilik gösteren süngerimsi polimerlerdir. Afinite destek malzemeleri ve doku iskeleleri olarak üstün kullanımlarının yanı sıra son yıllarda ilaç taşıyıcı malzemeler olarak da karşımıza çıkmaktadır. Moleküler baskılama yöntemi, kalıp molekül etrafında polimerik ağ oluşturularak hazırlanan bir tanıma tekniğidir. Bu yöntem saflaştırma ve ayırma işlemlerinde otuz yılı aşkın süredir kullanılmasına rağmen, ilaç salım çalışmalarında yüksek ilaç yükleme kapasitesi ve uzun süreli salım sağlayabilmesi nedeniyle büyük ilgi toplayan yeni bir yaklaşımdır. Bu çalışmada siprofloksasin baskılanmış 2-Hidroksietil metakrilat (HEMA) temelli *N-metakriloil-(L)*-histidin metil ester (MAH) kriyojeller [CIP-*p*(HEMAH)] sentezlenmiş ve karakterize edilmiştir. Siprofloksasin salım deneyleri gerçekleştirildikten sonra CIP-*p*(HEMAH) kriyojellerin *S. aureus, E. faecalis and E. coli*'ye karşı olan antibakteriyel aktiviteleri incelenmiştir. CIP-*p*(HEMAH) kriyojeller için kümülatif CIP salım oranları 6. ve 120. saatler için sırasıyla %80.6 ve %88.5 olarak bulunmuştur. CIP-*p*(HEMAH) kriyojellerin yara iyileştirme uygulamaları için umut vaat edici polimerik malzemeler olarak önerilebileceği sonucuna varılmıştır.

Anahtar Kelimeler

Siprofloksasin, moleküler baskılama, kriyojel, antimikrobiyal aktivite.

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INTRODUCTION

The volume of increased wound fluids and spreading of these exudates towards blood and other tissue parts stimulate microbial invasion [1]. Therefore, it would unavoidably emerge difficult-to-treat infections and prolong the healing time [2]. Both Gram-positive and Gram-negative bacterial strains are the most common causative agents of wound infections. It was reported that *Staphylococcus aureus, Enterococcus faecalis* and *Streptococcus pyogenes* are defined as Gram-positive, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* are presented as Gram-negative microorganisms for the pre-dominants associated with wound infections [3].

Cryogels have been extensively preferred support matrices and pave the way for many applications including the creation of wound dressings [4–6]. They have interconnected porous structure [7]. On the other hand, they could be prepared as biocompatible materials with the advantages of mechanical strength, and non-toxicity by selecting proper functional monomers [8,9]. HEMA (2-hydroxyethyl methacrylate) based polymers are one of the most recommended ones [10] due to their unique properties of providing high oxygen diffusion, water transport [11] and high biocompatibility [12]. Furthermore, polyHEMA (*p*HEMA) based materials could be synthesized by the incorporation of antimicrobial agents proving a significant advantage in the controlled release of the interested antimicrobial agent [13–15]. Ciprofloxacin has been introduced as a broad-spectrum antimicrobial agent having bactericidal effect against both to Gram-positive and Gram- negative bacterial strains [16]. It was assumed that the combination of ciprofloxacin into the *p*HEMA cryogels will enable obtaining an advantage to control developing infections with their antimicrobial property.

Molecular imprinting is a biomimetic way proven to create molecularly imprinted polymers (MIPs) having potential in the field of drug release studies [17]. These polymers ensure to obtain specific affinity regions for the target antimicrobial agent providing high drug loading capacity [18]. Molecular imprinting approach is based on polymerization process performed with functional monomers, crosslinkers, template molecules, and initiator [19]. After polymerization, recognition sites complementary to the template molecules in size, shape and functionality were created by the removal of template [20,21]. This technology have recently gained great attention with the properties of stability, cost-effectiveness and easy synthesis [17].

In the present study, imprinting was performed to prepare ciproflaxocin (CIP) imprinted 2- Hydroxyethyl methacrylate (HEMA) based *N*-methacryloyl-(*L*)-histidine methyl ester (MAH) containing [CIP-*p*(HEMAH)] cryogels. CIP-*p*(HEMAH) cryogels were characterized by Fourier Transform Infrared Spectrum (FTIR), scanning electron microscope (SEM) and swelling tests. The in-vitro CIP release studies were carried out to evaluate the applicability of CIP-*p*(HEMAH) cryogels. Finally, antimicrobial assays were performed to determine the antimicrobial ability of CIP-*p*(HEMAH) cryogels against *Staphylococcus aureus, Enterococcus faecalis* and *Escherichia coli.*

MATERIALS and METHODS

Materials

2- hydroxyethyl methacrylate (HEMA), *N,N'*-methylenebisacrylamide (MBAAm), *N,N,N',N'*-tetramethylene diamine (TEMED) and ciprofloxacin was purchased from Sigma (St. Louis, USA). The bacteria used for antimicrobial activity; *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Enteroccoccus faecalis* ATCC 29212 were obtained from the Culture Collection Department of Biology (Laboratory of Biotechnology), Hacettepe University, Turkey. Experiments were carried out three times, unless otherwise stated.

Preparation of ciprofloxacin imprinted cryogel

The functional monomer, *N*-methacryloyl-*L*-histidine (MAH) synthesized as the previous study [22]. Ciprofloxacin and MAH were mixed with a magnetic stirrer overnight at a ratio of 1:2 to form pre-complex. Then, as the monomer solution, the monomer (HEMA) and the crosslinker (MBAA) were prepared in deionized water (15 mL) to have a final monomer concentration of 15%. Precomplex solution was added to this monomer solution and mixed. Then, 1% (w/v) APS and TEMED were added to this solution, to initiate polymerization. The resulting solution was polymerized between two 20x20 cm glass plates at -12°C overnight. After the polymerization was completed, the cryogels reached room temperature and cut into circles of 0.8 mm diameter. Non-imprinted cryogels were produced following the similar methodology that of the CIP-*p*(HEMAH) cryogels in the absence of CIP imprinting and abbreviated as *p*(HEMAH)

cryogels. Imprinting efficiency was determined with the application of *p*(HEMAH) cryogels in the antimicrobial activity assays for the same microorganisms tested by CIP-*p*(HEMAH) cryogels.

Template and unreacted monomers were removed by washing CIP-*p*(HEMAH) and *p*(HEMAH) cryogels in methanol and deionized water for five days with changing the washing solutions in certain intervals.

Characterization studies

CIP-p(HEMAH) and *p*(HEMAH) cryogels were characterized by FTIR, SEM and swelling tests. All cryogels were freeze-dried in a lyophilizer (Christ Alpha LD) for 24 h before characterization.

MAH monomer, CIP-*p*(HEMAH) and *p*(HEMAH) cryogels examined by FTIR (FTIR 8000 Series, Shimadzu, Japan). The attenuated total reflectance (ATR) polarization unit was used for the samples. Dry samples were measured in the 4000-400 cm-spectral wavelength range.

Surface morphology and pore structure of CIP-*p*(HEMAH) and *p*(HEMAH) cryogels were examined by SEM. The cryogel samples were covered with a thin layer of gold before being placed in the sample holder.

Dry cryogels were first weighed (W_0) to determine the equilibrium swelling degree of CIP-*p*(HEMAH), *p*(HEMAH) cryogels. The cryogels were then dropped into 100 ml of distilled water and the swollen cryogels were weighed (W_{sw}) at regular intervals. The swelling degree of CIP- *p*(HEMAH), *p*(HEMAH) and *p*(HEMA) cryogels was calculated as Equation 1.

$$Swelling\ degree = \frac{W_0 - W_{sw}}{W_0}$$

Eq. (1)

To calculate the macropore percentage, the fully swollen cryogels were squeezed and weighed (W_{sq}). After removing water from large pores, the percentage of macropore was calculated using Eq. 2.

$$Macroporosity\ \% = \frac{W_{sw} - W_{sq}}{W_{sw}} x100$$

Eq. (2)

Cryogel weights were recorded in grams. All experiments were done three times and their averages were taken.

Loading and in vitro ciprofloxacin release studies

Drug loading experiments were performed by placing CIP-*p*(HEMAH) cryogels into CIP solutions (10 mg/mL) at 25°C for 24 h. The amount of loaded CIP was determined by measuring the initial and final CIP concentrations at 270 nm using UV-Vis Spectrophotometer (Shimadzu, Japan).

In vitro drug release experiments were performed using 2 mL pH 7.4 phosphate buffer saline (PBS) at 37°C. At certain times, after the release medium was taken for measurement, new buffer solution was added in its place. The amount of ciprofloxacin released was analyzed by UV-Vis Spectrophotometer (Shimadzu, Japan) at 270 nm. Cumulative release (%) was determined using Equation 3:

$$Cumulative\,release\ \% = \frac{W_{total\ release}}{W_{total\ drug\ amount}} x100$$

Eq. (3):

Antimicrobial assay

The antimicrobial performances of CIP-*p*(HEMAH) and *p*(HEMAH) cryogels were determined by agar disc diffusion method. For this purpose, *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *E. faecalis* ATCC 29121 strains were inoculated in Luria Bertani broth and incubated at 37°C overnight. These fresh cultures were used for the preparation of 0.5 McFarland turbidity 1.5×10^8 CFU/mL) by suspending bacterial cells. Then, the samples (100 µL) from these bacterial suspensions were inoculated in Mueller Hinton Agar (MHA) plate and incubated at 37°C for 18h. After incubation, the antimicrobial performances of CIP-*p*(HEMAH) and *p*(HEMAH) cryogels were determined by measuring the diameters of the inhibition zones in cm. The antibacterial tests were performed in triplicate.

RESULTS and DISCUSSION

Cryogels loaded with antimicrobial agents have recently gained great attention due to their potential for sustained drug release in a controllable manner [23]. Drug imprinted super porous cryogels meet the antibacterial drug delivery requirements. CIP was selected as an antibacterial template for the preparation of binding regions which is complementary to CIP onto the resultant polymeric matrix. For the synthesis of MIPs in this study, HEMA and MAH were applied as an assistant and functional monomer, respectively. These monomers have been commonly used for the construction of MIPs with multiple functionalities. Hydrophilic polymer networks that can be synthesized and purified in water are preferred over those requiring organic solvents. A hydrophilic surface also improves biocompatibility and prevents microorganisms from adhering [24].

In general, MIPs can serve as pre-designed recognition materials for template, however they also have adjustable properties and promising abilities such as responding against the stimulus, providing the signal transduction and increased drug loading capacity, designing purification and separation systems, creating affinity sensing platforms [25]. It is supposed to occur non-covalent interactions between functional monomers and CIP.

Molecular imprinting technology is a new approach in the development of new technologies to optimize drug delivery in recent years. MIPs can remain stable against a variety of enzymatic activities. They also preserve their structure in a wide range of physical conditions. In addition to their mechanical stability, it is also possible to control the drug release rate by changing the monomer/template molecule ratio [26].

Characterization studies

FTIR analysis was performed to show the type of chemical bonds found in the CIP-*p*(HEMAH) and *p*(HEMAH) cryogels (Figure 1). The characteristic stretching bands hydrogen-bonded alcohol, OH, around 3306 and 3243 cm^{-1} indicated for CIP-*p*(HEMAH) and *p*(HEMAH) cryogels, respectively. Carbonyl stretching (amide I) at 1723 and 1717 cm^{-1} was shown for CIP-*p*(HEMAH) and *p*(HEMAH) cryogels, respectively. CN and NH stretching peaks at 1526 cm^{-1} can be attributed to the vibration of amide II. The peaks at 1652 and 1655 cm^{-1} can be assigned to amide bands for CIP-*p*(HEMAH) and *p*(HEMAH) cryogels, respectively [13]. The peaks at 1385 and 1387 cm^{-1} (aromatic ring) and at 1069 and 1071 cm^{-1} (imidazole ring) can be allocated to the presence of MAH. When these obtained results were taken into consideration, it is clearly seen that MAH was successfully incorporated into prepared cryogels as a functional monomer.

SEM images of CIP-*p*(HEMAH) and *p*(HEMAH) cryogels were given in Figure 2A and 2B respectively. CIP-*p*(HEMAH*)* and *p*(HEMAH) cryogels were synthesized with interconnected pores having thin polymeric wall. The porous network structure of these cryogels enables mass transfer of the fluids, therefore, CIP-*p*(HEMAH) cryogels showed high absorption ability. By the way,

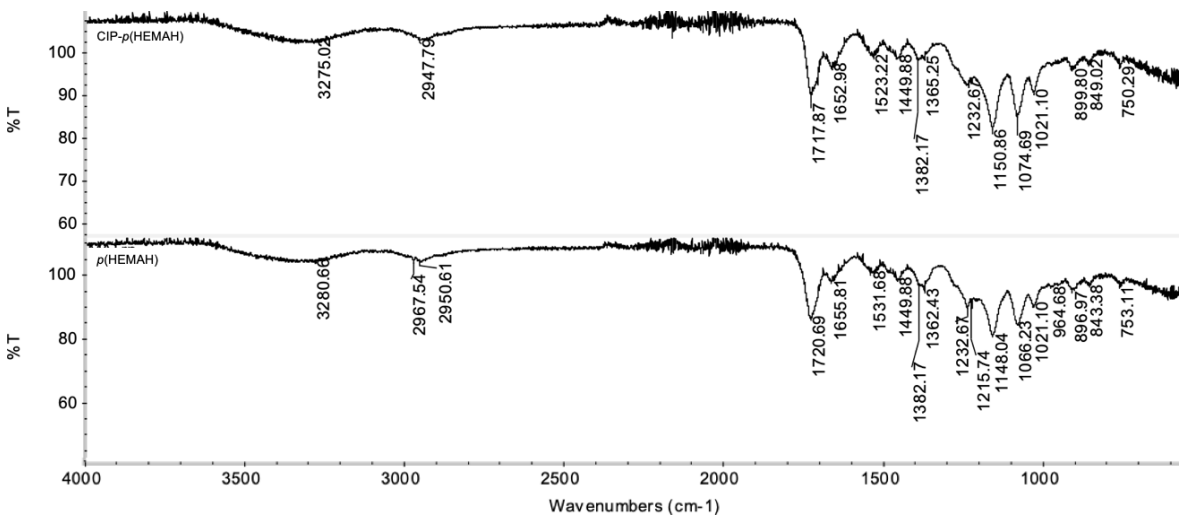

Figure 1. The FTIR analysis of CIP-*p*(HEMAH) and *p*(HEMAH) cryogels.

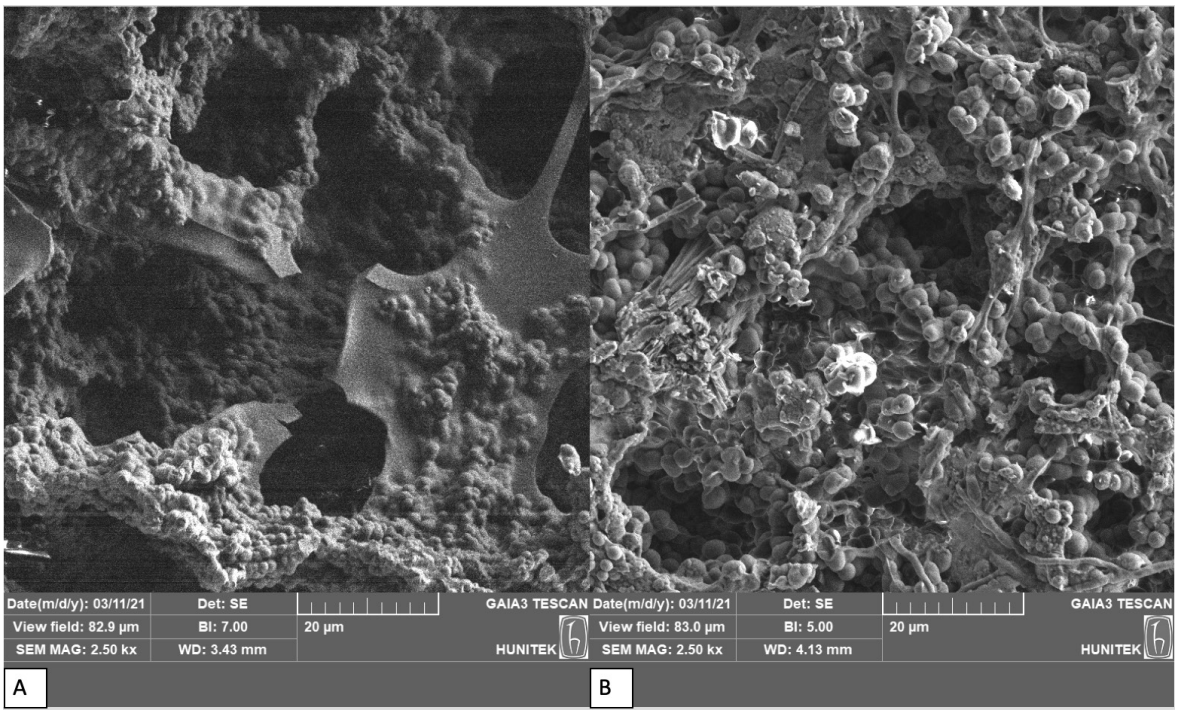

Figure 2. SEM images of (A) CIP-p(HEMAH) and (B) p(HEMAH) cryogels.

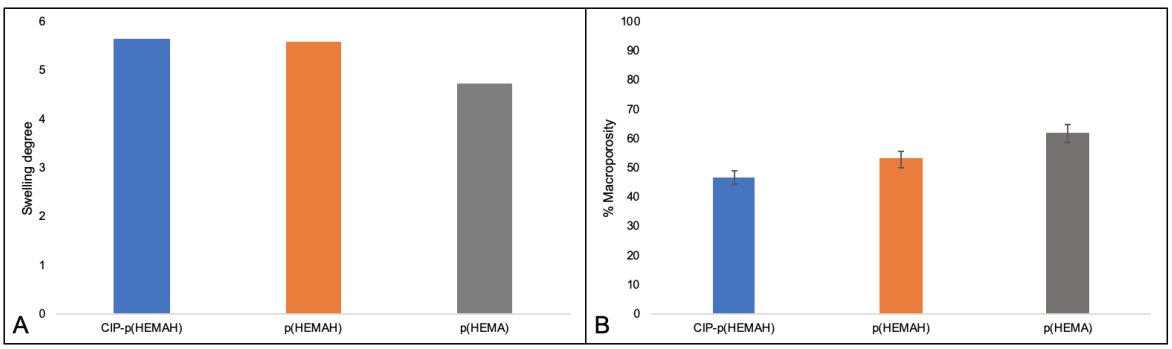

Figure 3. The (A) swelling degree and (B) macroporosity of CIP-*p*(HEMAH), p(HEMAH) and *p*(HEMA) cryogels.

CIP-*p*(HEMAH) cryogels provide an opportunity to release CIP. When all these properties were taken into consideration, CIP-*p*(HEMAH) cryogels are good candidates in the construction of medical support materials. It was demonstrated that the diameters of CIP-*p*(HEMAH) and *p*(HEMAH) cryogels were found to be 17±5 and 20±5 µm, respectively.

The swelling degree of fabricated cryogels is related to water uptake capacity of their spongy-like structure. The CIP-*p*(HEMAH), the *p*(HEMAH) and *p*(HEMA) cryogels indicated good swelling properties having high swelling degrees in swollen states. Cryogels with large and connected pores also having linked macroporous structure increases the mechanical strength of the biosorbent. The swelling degree and macroporosity of CIP-*p*(HEMAH), the *p*(HEMAH) and *p*(HEMA) cryogels were shown in Figure 3A and 3B, respectively. The swelling degree of CIP-*p*(HEMAH), the *p*(HEMAH) and *p*(HEMA) cryogels were found as 5.6, 5.5 and 4.7, respectively. Besides, the macroporosity ratios of CIP-*p*(HEMAH), the *p*(HEMAH) and *p*(HEMA) cryogels were found as 47, 53, and 62, respectively. The obtained macroporosity values indicate that a transition occurs between the inner and outer surfaces of the matrix due to the interconnected macropores allowing CIP to be released out of the cryogel.

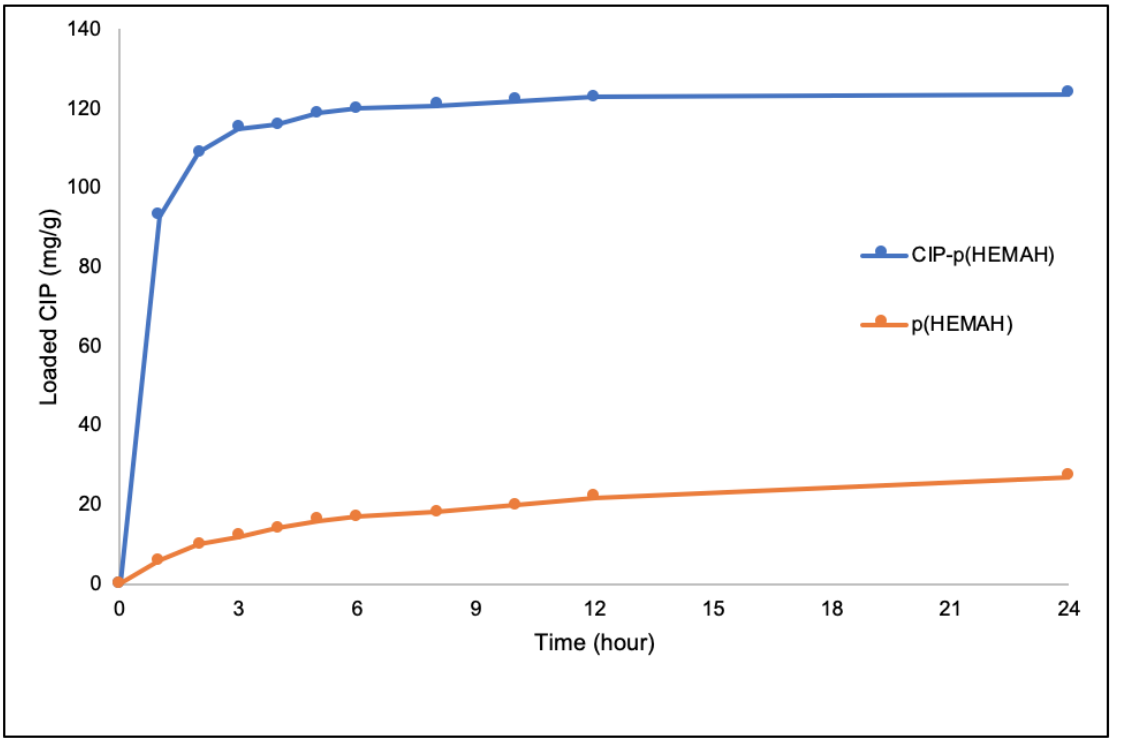

Figure 4. CIP loading capacities of CIP-*p*(HEMAH) and *p*(HEMAH) cryogels.

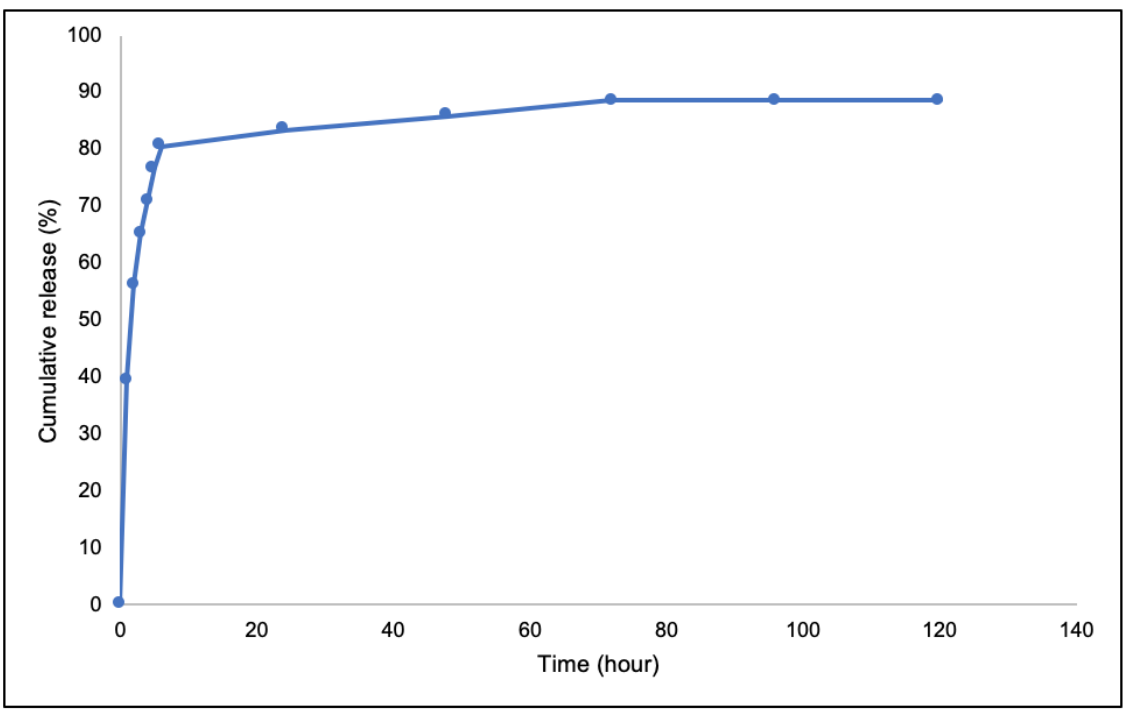

Figure 5. The cumulative release curve of CIP-*p*(HEMAH) cryogels.

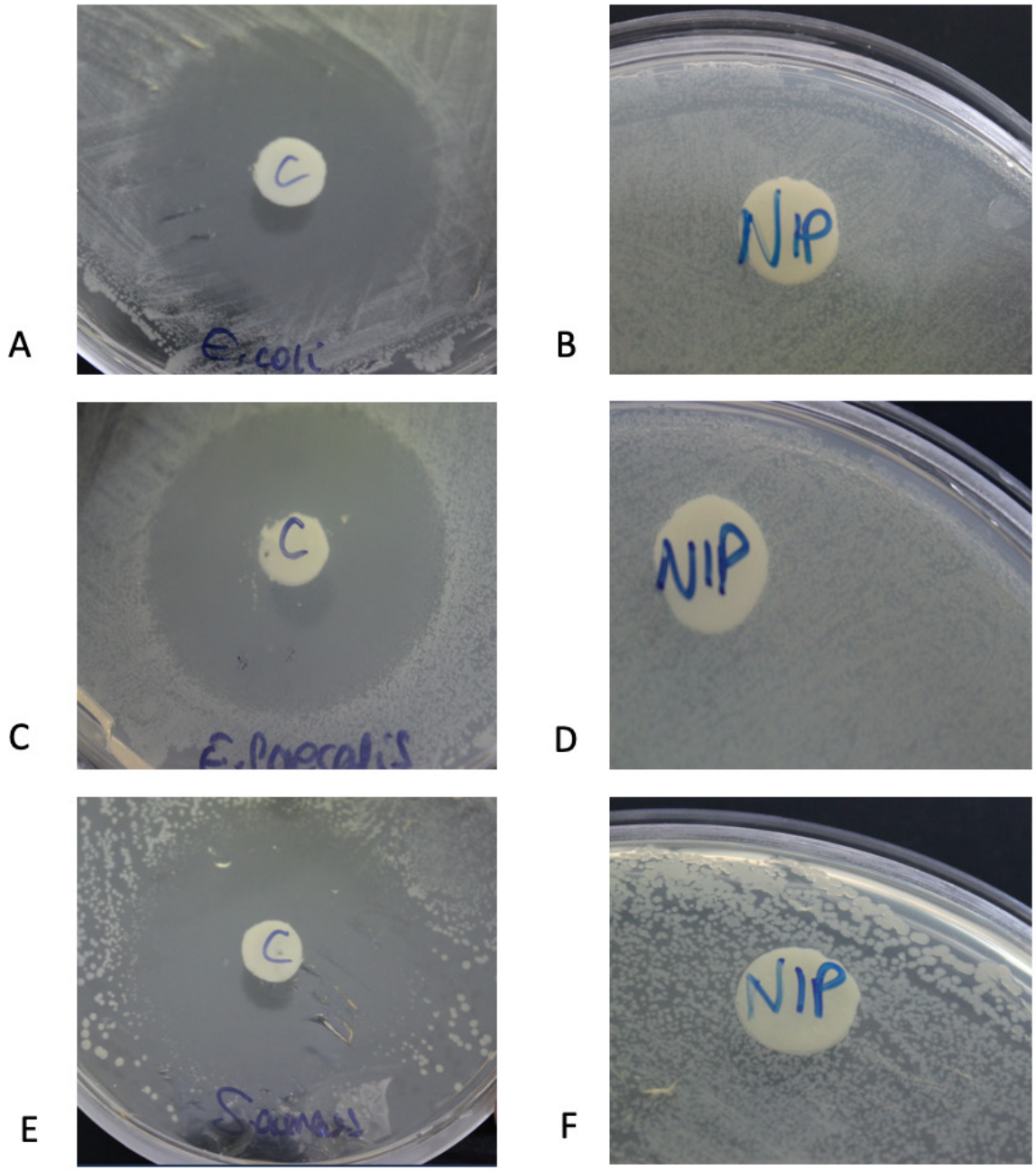

Figure 6. Antimicrobial performances of CIP-*p*(HEMAH) cryogels against *(A) E. coli, (C) E. faecalis, (E) S. aureus;* Antimicrobial performances of *p*(HEMAH) cryogels against *(B) E. coli, (D) E. faecalis, (F) S. aureus.*

Loading and in vitro ciprofloxacin release studies

The amount of loaded CIP into CIP-*p*(HEMAH) and *p*(HEMAH) cryogel was determined by spectrophotometer [27]. The maximum loaded CIP was calculated as 123.8 mg/g and 27.1 mg/g for CIP-*p*(HEMAH) and *p*(HEMAH) cryogels, respectively (Figure 4). The amount of loaded CIP into CIP-*p*(HEMAH) was higher than that of *p*(HEMAH) cryogels and therefore, it can be concluded that efficient imprinting was carried out and selective binding cavities for CIP was successfully produced.

The result of in vitro CIP release experiments performed using CIP-*p*(HEMAH) was shown in Figure 5. It was found that cumulative CIP release ratios were indicated as 80.6% and 88.5% for CIP-*p*(HEMAH) cryogels at the end of 6 and 120 h, respectively. Several potential binding regions have the ability to enhance affinity of CIP-*p*(HEMAH) cryogels against. On the other hand, cumulative CIP release ratio was found as 98.5% for *p*(HEMAH) cryogels at the end of 3 h, this reason can be attributed to the non-specific interactions between CIP and *p*(HEMAH) cryogels.

Antimicrobial assay

The antimicrobial performances of CIP-*p*(HEMAH) and *p*(HEMAH) cryogels were determined using both Gram-positive and Gram-negative bacteria. In this respect, *S. aureus* and *E. faecalis* were included as Gram-positive bacterial strains, whereas *E. coli* was preferred as a model for Gram-negative bacterial strain. The antimicrobial tests were carried out by evaluating the diameters of inhibited growth zones. The obtained inhibition zones around CIP-*p*(HEMAH) cryogels for *E. coli*, *E. faecalis* and *S. aureus* were shown in Figure 6A, 6C and 6E, respectively. As expected, there was no inhibition zone around *p*(HEMAH) cryogels for *E. coli*, *E. faecalis* and *S. aureus* as seen in Figure 6B, 6D and 6F, respectively. It was revealed that the antimicrobial effect of CIP-*p*(HEMAH) cryogels was originated from the successful CIP imprinting. It is noteworthy to indicate that the inhibition zones were substantially attributed to the usage of CIP-p(HEMAH) cryogels. The diameters of inhibition zones were measured as 3.5 ± 0.4, 3.8 ± 0.5 and 4.0 ± 0.7 cm for *S. aureus, E. faecalis* and *E. coli*, respectively. It could be clearly seen that CIP imprinted p(HEMAH) cryogels possess high antimicrobial performance.

The molecular imprinting provides to enhance the drug release period with the advantage of resulting to inhibit bacterial growth. The antibacterial performance of the released CIP was verified with obtained inhibition zones using CIP loaded cryogels against *S. aureus*, *E. faecalis* and *E. coli.* In literature, there have been some publications related to drug loaded materials developed for sustained drug release.

To give an example, Kioomars et al. [27] prepared CIP imprinted HEMA and methacrylic acid (MAA) based hydrogels and optimized the polymerization conditions to improve ocular drug delivery. Optimization experiments were performed to increase CIP loading capacity of prepared CIP imprinted hydrogels. The efficacy of releasing CIP was examined using artificial tears. Besides, the antibacterial effect of CIP imprinted hydrogels was indicated against *P. aeruginosa* and *S. aureus*. It can be concluded that CIP imprinted hydrogels have the ability to release CIP effectively from hydrogel discs with the advantage of any important side effect [27].

Silva et al. [28] produced moxifloxacin (MXF) imprinted silicone hydrogels designed as contact lens material using acrylic acid as co-monomer. It was aimed to establish long-term release by loading high amounts of MXF on molecularly imprinted silicone hydrogels. They prepared MXF imprinted hydrogels using different amounts of co-monomers, as a result, they reported that the hydrogel containing the highest amount of co-monomer was released MXF from the hydrogels for a period of approximately 2 weeks. Furthermore, the antibacterial effect of MXF imprinted hydrogels was investigated against *S. aureus* and *S. epidermidis* by agar diffusion test. Accordingly, the highest diameters of the obtained inhibition zone were found as 67±4 mm and 59±6 mm for *S. aureus* and *S. epidermidis*, respectively [28].

Apart from cryogels, nanocarriers have received great interest in the field of drug delivery providing to develop well-targeted drug delivery and maintaining drug stability during releasing duration [3]. Kempe et al. [29] synthesized imprinted methacrylic acid and trimethylolpropane trimethacrylate based nanocarriers for erythromycin release. Selectivity of the erythromycin recognition sites was confirmed with competitive structurally different drug molecules. The results showed 87% loading efficiency and following the initial burst seen during the first day 82% release was pointed out after a week [29].

One of the other examples of the developed materials for antibiotic release by molecular imprinting technique is bacterial nanofiber modified with gentamicin imprinted microparticles which were conducted by our research group. For revealing the effective gentamicin releasing, optimization studies were performed during preparation of gentamicin imprinted bacterial nanofibers. As a consequence, ratios of composite nanofibers containing three different rates of co-monomers have achieved cumulative release in the range of approximately 97%-60%. In addition, antimicrobial activity of gentamicin imprinted microparticles was tested against *E. coli* and *S. aureus* using disk diffusion test, and therefore, inhibition zone diameters were reported in the range of 7.5-11 and 12.5-14.5 mm for *E. coli* and *S. aureus*, respectively [30].

CONCLUSION

Cryogels, spongy polymers, are effective to role as swelling and simultaneously drug release materials. Besides, cryogels can be prepared for specific drugs. The strategies developed for drug release are likely to also be useful to improve in-vivo experiments. In this study, *p*(HEMAH) cryogels were prepared with the incorporation of CIP imprinting. Molecular imprinting was preferred to elevate CIP loading capacity of cryogels providing selective recogni-

tion cavities showing high affinity for CIP and maintaining controlled drug release. It was found that CIP-*p*(HEMAH) cryogels indicated burst release of 65% within 3 h. It can be concluded that CIP-*p*(HEMAH) cryogels have been introduced as effective promising materials for sustainable drug release due to their verified ability in CIP releasing over a prolonged time period. Antibacterial performances were confirmed using an agar disc diffusion test by evaluating the antibacterial activity of CIP-*p*(HEMAH) cryogels against S. *aureus, E. faecalis* and *E. coli.* As a consequence, CIP-*p*(HEMAH) cryogels could be recommended as potential well-generated polymeric matrices for wound healing applications for the future perspectives.

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