S. Aslıyüce et al. / Hacettepe J. Biol. & Chem., 2022, 50 (2), 205-214

Research Article

Hacettepe Journal of Biology and Chemistry

journal homepage: www.hjbc.hacettepe.edu.tr

Cefuroxime Imprinted p(HEMATrp) Cryogels: Preparation, Characterization and Antibacterial Role

Sefuroksim Baskılanmış p(HEMATrp) Kriyojeller: Hazırlanması, Karakterizasyonu ve Antibakteriyel Rolü

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ABSTRACT

Both Gram-negative and positive bacterial strains are known as the most frequently responsible causative agents for wound infections. These infections can resulted in morbidity and mortality due to the severity. Antimicrobial agents have often been preferred to treat these infections. In this respect, Cefuroxime (CXM) belongs to the second-generation cephalosporins could be suggested against wound infections. In recent years, the designing of drug delivery systems has received interest and cryogels are promising tools for creating these systems. Their elastic nature, high macroporosity, absorption and releasing ability make these materials unique for drug delivery. Besides, the imprinting approach could be integrated into cryogelation and the resultant matrix can recognize target antimicrobial agent having high selectivity and sensitivity prepared along with an easy and cost-effective methodology. In the present study, CXM was imprinted onto Hydroxyethyl methacrylate (HEMA) based N-methacryloyl-l-tryptophan (MATrp) containing [p(HEMATrp)] cryogels. MATrp was used as the co-monomer for the preparation of CXM-p(HEMATrp) cryogels. Characterization experiments were performed to analyze the structure of prepared cryogels. Following drug loading and releasing assays, antimicrobial performances of CXM-p(HEMATrp) cryogels was investigated against *Staphylococcus aureus, Enterococcus faecalis* and *Escherichia coli*. In conclusion, CXM-p(HEMATrp) cryogels have been recommended as potential carriers for further biomedical applications.

Key Words

Cefuroxime, molecular imprinting, cryogels, antimicrobial activity.

ÖZ

Hem Gram negatif hem de Gram pozitif bakteriler, yara enfeksiyonlarından en sık sorumlu etkenler olarak bilinirler. Bu enfeksiyonlar, ciddiyetine bağlı olarak morbidite ve mortaliteye neden olabilirler. Söz konusu enfeksiyonları tedavi etmek için sıklıkla antimikrobiyal ajanlar tercih edilmektedir. Bu bakımdan ikinci kuşak sefalosporinlerden olan Cefuroxime (CXM) yara enfeksiyonlarına karşı önerilebilir. Son yıllarda, ilaç taşıma sistemlerinin tasarımı ilgi görmekte olup, kriyojeller bu sistemleri oluşturmak için umut verici araçlardır. Elastik yapıları, yüksek makro gözeneklilikleri, emilim ve salım kabiliyetleri, bu malzemeleri ilaç taşıma için benzersiz kılmaktadır. Ayrıca, moleküler baskılama yaklaşımı kriyojelasyona entegre edilebilir ve elde edilen matriks, kolay ve uygun maliyetli bir metodoloji ile birlikte hazırlanmış olup, yüksek seçicilik ve hassasiyetle hedef antimikrobiyal ajanı tanıma yeteneğine sahiptir. Bu çalışmada, CXM, Hidroksietil metakrilat (HEMA) temelli N-metakriloil-l-triptofan (MATrp) içeren [p(HEMATrp)] içeren kriyojellere baskılanmıştır. MATrp, CXM-p(HEMATrp) kriyojellerinin hazırlanmasında ko-monomer olarak kullanılmıştır. Hazırlanan kriyojellerin yapısını analiz etmek için karakterizasyon deneyleri yapılmıştır. İlaç yükleme ve salım çalışmalarının ardından, CXM-p(HEMATrp) kriyojellerin *Staphylococcus aureus, Enterococcus faecalis* ve *Escherichia coli* 'ye karşı antimikrobiyal performansları incelenmiştir. Sonuç olarak, CXM-p(HEMATrp) kriyojeller, ileri biyomedikal uygulamalar için potansiyel bir taşıyıcı olarak önerilmektedir.

Anahtar Kelimeler

Sefuroksim, moleküler baskılama, kriyojel, antimikrobiyal aktivite.

Article History: Received: Apr 20, 2021; Revised: May 20, 2021; Accepted: Jul 27, 2021; Available Online: Feb 28, 2022.

DOI: https://doi.org/10.15671/hjbc.923371

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INTRODUCTION

The issue of improving the effectiveness of wound healing with the antimicrobial agents used today has been the interesting focus of both researchers and clinicians [1]. The advances in drug targeting and delivery systems are presently under development focusing on remodeling [2]. It is expected that ideal novel developments of materials with the ability to deliver drugs will bring new hopes for improvement studies [3].

Wounds could be existed as burns, ulcers, open surgical types including incisions have been maintained as one of the most serious clinical problems in terms of protecting the sterility of the wound [4,5]. Almost any type of bacteria such as *Staphylococcus aureus, Enterococcus faecalis* and *Escherichia coli* have usually been stated as responsible strains for wound infections [5]. In this respect, it is necessary to produce new aspects for the development of dressings integrated with appropriate antibacterial agents to take measurements and prevent the infections.

The support materials called also as carriers to *release* antimicrobial agents have significant actions for release performances, loading capacity, and biocompatibility. Polymeric materials could be prepared as microcapsules [6], microbeads [7], nanocarriers [1] such as nanoparticles [8], nanocapsules [9], nanotubes [10], nanogels [11], nanofibers [12], hydrogel [13], cryogels [14] including composite [15] and injectable microcryogel [16] forms for the formulation of drug delivery platforms. As one of the effective polymeric carriers, cryogels can be prepared under sub-zero temperatures and freezing conditions have an important effect to produce desirable mechanical property [17]. Cryogels have interconnected porous structure enabling cell filtration and elastic network providing advanced swelling ability and improved drug preservation over their traditional counterparts [18]. Besides, the ease of controlling pore sizes [19] and their absorption ability provide the diffusion of the exudate [20]. The resultant polymeric matrix has desirable physical features and attractive both drug loading and releasing characteristics which can be obtained by using various co-monomers. By the way, when it is considered that each implementation requires a specific property such as releasing the drug at a certain time obtained by intended functionalization, cryogels have been specifically established materials [21]. It is also indicated that cryogels have been popularly preferred for diversified applications due to their verified better and faster healing ability [22]. Besides, control over releasing is succeeded by the optimization experiments performed in designing cryogels. Hydroxyethyl methacrylate (HEMA) based polymers (pHEMA) offers opportunity in designing medical devices with the application of various copolymer compositions [23]. In the related literature, it was reported that pHEMA based materials were favorable due to their ability to inhibit fluid loss and quicken wound healing.

Molecularly imprinted polymers (MIPs) are exploited as promising matrices for the preparation of drug delivery devices and the researches in this area is still developing [24]. MIPs, also known as artificial polymers, can be synthesized as co-polymers by using functional/assistant monomers and cross-linkers. The concentrations of these components affect the morphology and functional structure of the resultant matrix and complementary cavities specific for the template [25]. The main reason for the application of MIPs in drug release can be attributed to the possibility of enhancing the interactions between target agent and their formed recognition cavities into the polymeric matrix [26]. Therefore, one of the most well-known characteristics of polymeric structures serving for drug release could be obtained by releasing the agent over an extended period of time in a regulated manner [27]. Cefuroxime (CXM) belongs to the second-generation cephalosporins and acts against both Gram-positive and Gram-negative microorganisms [28]. It is reported that antibacterial role of cefuroxime for Gram negative strains is slightly higher than that for Gram positive ones [29]. In literature, it was reported that the effect of CXM as a treatment option for the post-surgical infections [30].

The present study aimed to perform the synthesis of HEMA based *N*-methacryloyl-*l*-tryptophan (MATrp) containing cryogels [p(HEMATrp)] by the incorporation with CXM using imprinting technology. The characterization studies of CXM imprinted p(HEMATrp) [CXM-p(HEMATrp)] cryogels were carried out by Fourier Transform Infrared Spectrum (FTIR), scanning electron microscope (SEM), and swelling tests. After drug loading the step, drug release experiments were studied in-vitro to examine the efficiency of CXM-p(HEMATrp) cryogels. Besides, antimicrobial potentials of CXM-p(HEMATrp) cryogels were evaluated by agWar disc diffusion assay against *S. aureus, E. faecalis and E. coli.*

MATERIALS and METHODS

Materials

HEMA, *N,N'*-methylenebisacrylamide (MBAAm), *N,N,N',N'*-tetramethylene diamine (TEMED) and cefuroxime were purchased from Sigma (St. Louis, USA). Phosphate buffer saline (PBS), NaOH and Na_2CO_3 were purchased from Merck. MATrp was obtained from Laboratory of Biochemistry, Department of Chemistry, Hacettepe University, Turkey. *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Enterococcus 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.

Synthesis of CXM-p(HEMATrp) and p(HEMATrp) cryogels

MATrp was used as the co-monomer for the preparation of CXM-p(HEMATrp) cryogels. CXM and MATrp were mixed overnight in a magnetic stirrer to form a precomplex with the ratio of 1:3 (mmol). HEMA (2.6 mL) was also dissolved in deionize water (10 mL). The precomplex solution was then added to the HEMA solution and mixed for 2 h. In a separate beaker, MBAAm (0.566 g) was dissolved in deionized water (20 mL). Then, HEMA-precomplex and MBAAm solutions were mixed in a single beaker. TEMED (50 mL) and APS (30 mg) were added into the final solution to initiate polymerization. Thin strips of 0.1 cm thickness were placed between two glass plates (20x20 cm). Three edges of these glass plates were tightly sealed to prevent flowing of the solution. Prepared polymer solution was quickly poured between these two glass plates and allowed to polymerize at -12 °C for 24 h. After the polymerization was completed, the cryogels taken to room temperature were cut into discs of 0.8 cm in diameter. p(HEMATrp) cryogels were prepared using similar procedure as that of CXM-p(HEMATrp) cryogels without containing CXM. p(HEMA) cryogels were prepared by the similar method except containing CXM and MATrp. The CXM-p(HEMATrp), p(HEMATrp) and p(HEMA) cryogels were washed with deionized water for 2 h.

Characterization of CXM-p(HEMATrp) and p(HEMATrp) cryogel discs

Before characterization studies, the CXM-p(HEMATrp), p(HEMATrp) and p(HEMA) cryogels were dried in a lyophilizer (Christ Alpha LD).

Dry cryogels were first weighed to examine the swelling properties of CXM-p(HEMATrp), p(HEMATrp) and p(HEMA) cryogels (W_0). Cryogels were then left in a beaker containing 200 mL of distilled water and weighed at certain times between 1-120 min. Dry cryogels were first weighed to examine the swelling properties of cryogels. The weight of the maximum swollen cryogel was recorded (W_{sw}) to determine the macroporous percentage of the cryogels. Then the cryogels were squeezed to remove water from the macroporous and weighed (W_{sq}). The macroporous percentage and the water retention of the p(HEMA), CXM-p(HEMATrp) and p(HEMATrp) cryogels were calculated by Equation 1 and Equation 2, respectively.

$$Macroporosity\ \% = \frac{W_{sw} - W_{sq}}{W_{sw}} x100 \qquad \text{Eq(1)}$$

$$Water\ retention\ \% = \frac{W_0 - W_{sw}}{W_0} x100 \qquad \text{Eq(2)}$$

SEM images were taken to observe the pore structure and size of the CXM-p(HEMATrp) and p(HEMATrp) cryogels. Dry cryogel samples were plated with thin layer gold before imaging.

FTIR measurements were performed in the spectral wavelength range of 4000-400 cm^{-1} in order to detect the bonds in the polymeric structure of CXM-p(HEMATrp) and p(HEMATrp) cryogels.

Cefuroxime removing, loading and releasing studies

CXM-p(HEMATrp) cryogels were treated with CXM solution (10 mg/mL) prepared in PBS at 37 °C for 24 h to reload the CXM on to the CXM-p(HEMATrp). The amount of CXM loaded onto the cryogels was determinate by visualizing change of the CXM concentration in the solution measured at 281 nm with a UV-vis spectrophotometer.

Drug release experiments were performed in vitro at 37°C for 5 days. 5 mL of pH 7.4 PBS was used as the release medium. The amount of drug released was determined by measuring CXM in the buffer solution at 281 nm using a UV-Vis Spectrophotometer. The buffer solution was replaced with a new one after each measurement. Cumulative release (%) was determined by Equation 3:

$$Cumulative\,release\ \% = \frac{W_{total\ release}}{W_{total\ drug\ amount}} x100 \quad \text{Eq(3)}$$

Antimicrobial assay

Agar disc diffusion method was applied to investigate the antibacterial activity of CXM-p(HEMATrp) and p(HEMATrp) cryogels. *E. coli*, *S. aureus*, *E. faecalis* strains were included to evaluate the antibacterial effectiveness of CXM-p(HEMATrp) cryogels in a broad spectrum. In this respect, overnight cultures of test bacterial strains were prepared 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. Mueller Hinton Agar (MHA) was used to perform the agar disc diffusion test. Following inoculating the bacterial strains onto MHA plates, incubation was carried out at 37°C overnight. Finally, the antimicrobial potentials of CXM-p(HEMATrp) cryogels were evaluated according to the growth inhibition zones measured in cm. The antibacterial tests were performed in triplicate.

RESULTS and DISCUSSION

The recent literature overview demonstrates that researches have focused on the understanding and development of the drug including materials for the purpose of drug release. The findings of these works showed that drug loaded cryogels have been introduced as good alternatives for the development of different drug releasing strategies. Besides, the optimization of the cryostructuring process performed with changing monomer:crosslinker ratio, temperature and freezing conditions has a crucial role in preparing gel systems which are referred as controlled drug delivery devices. Imprinting approach aiming to generate molecular geometries contributes to alter the affinity of the polymeric-based drug release, providing quite simple and easy methodology. The imprinting strategy could be easily integrated to synthesize cryogels and their enviable properties combined with the advantages of imprinting make these materials potential devices for biomedical applications including drug delivery. Imprinting of antimicrobial agents has increased the applicability of drug containing cryogels due to their selective recognition ability of target agent [31].

Table 1. The comparative overview of the molecularly imprinted antibacterial materials applied in literature.

Drug	Monomer/ comonomer	Material	Loading capacity	Cumulative release (%)	Inhibition/reduction bacterial growth					R
					E. coli	*S. aureus*	*S. epidermidis*	*E. faecalis*	*P. aeruginosa*	
Gentamicin	MAA	Nanofiber	204.6-142.8 mg/g	97-60	12.5 mm	14.5 mm	-	-	-	[12]
Erythromycin	MAA	Nanocarrier	76 mg/g	82	-	-	-	-	-	[35]
Moxifloxacin	HEMA/AA	Hydrogel	9.8-64.9 µg/mg	up to 60	-	67±4 mm	59±6 mm	-	-	[36]
Ciprofloxacin	HEMA/AA	Hydrogel	170-210 µg/disc	~62-98	-	-	-	-	0.8 log cfu/mL	[37]
Azithromycin	MAA	Nanoparticle	127 mg/g	up to 98	-	-	-	-	-	[38]
Vancomycin	MAA	Alginate dressing	-	~90	-	-	-	-	-	[39]
Vancomycin	HEMA/ DEAEMA	Nanosphere	-	-	-	up to 92%	-	-	-	[40]
Cefuroxime	HEMA/ MATrp	Cryogel	138 mg/g	76	3.1 ± 0.2	2.9 ± 0.1	-	2.9 ± 0.2	-	In this study

MAA: methacrylic acid, HEMA: 2-hydroxyethyl methacrylate, AA: acrylic acid, DEAEMA: 2-(diethylamino) ethyl methacrylate, MATrp: N-methacryloyl-l-tryptophan.

Our research group aimed to fabricate molecularly imprinted cryogels for mitomycin-c delivery. For this purpose, mitomycin-c imprinted poly(2-hydroxyethyl methacrylate-*N*-methacryloyl-*l*-glutamic acid) cryogels were constructed and it was reported that the cumulative release of mitomycin-c affected by the concentration of crosslinker and loaded mitomycin-c [32]. In another study conducted by our research group again, Doxorubicin (DOX), an anthracycline agent applied as a chemotherapeutic, was imprinted poly(hydroxyethyl methacrylate-*N*-methacryloyl-(*l*)-glutamic acid methyl ester)–poly(ethylene glycol) diacrylate–gelatin (PHEMAGA-G) pH responsive cryogels [33].

Apart from the imprinting strategy, we prepared lysozyme integrated gelatin based microcryogels. Lysozyme loading and releasing experiments were performed and lysozyme releasing ability was clarified. The results showed that lysozyme loaded gelatin-microcryogels were indicated antibacterial activity against both Gram-positive and Gram-negative strains [16].

In a previous study, cryogels were fabricated for selective binding of tetracycline (TC) by embedding TC-imprinted poly(hydroxyethyl methacrylate-*N*-methacryloyl-*l*-glutamic acid methyl ester [p(HEMA-MAGA)] particles into PHEMA cryogel. Following characterization studies, aqueous solution of TC was used to perform adsorption assays, and adsorption abilities were examined in different experimental conditions. The maximum TC adsorption capacity was calculated as to 680 mg TC/g at room temperature. It was reported that prepared composite cryogels are efficient at recognizing TC in real samples with high selectivity [34]. The comparative overview of the molecularly imprinted antibacterial materials applied in literature is given in Table 1.

Characterization

The water retention percentages of p(HEMA), CXM-p(HEMATrp) and p(HEMATrp) cryogels were calculated as 758%, 560% and 440%, respectively (Figure 1). When comparing p(HEMA) cryogels with p(HEMATrp) and CXM-p(HEMATrp), the water retention of p(HEMATrp) and CXM-p(HEMATrp) was lower due to the hydrophobic nature of MATrp. The difference between p(HEMATrp) and CXM-p(HEMATrp) is thought to be caused by cavities formed by molecular imprinting. The macroporosities of p(HEMA), p(HEMATrp) and CXM-p(HEMATrp) cryogels were reported as 74%, 71% and 69%, respectively.

SEM images of CXM-p(HEMATrp) and p(HEMATrp) cryogels are presented in Figure 2. According to this figure, indicated that the pore diameters of p(HEMATrp) and CXM-p(HEMATrp) cryogels are about 65±5 (Figure 2A) and 55±5

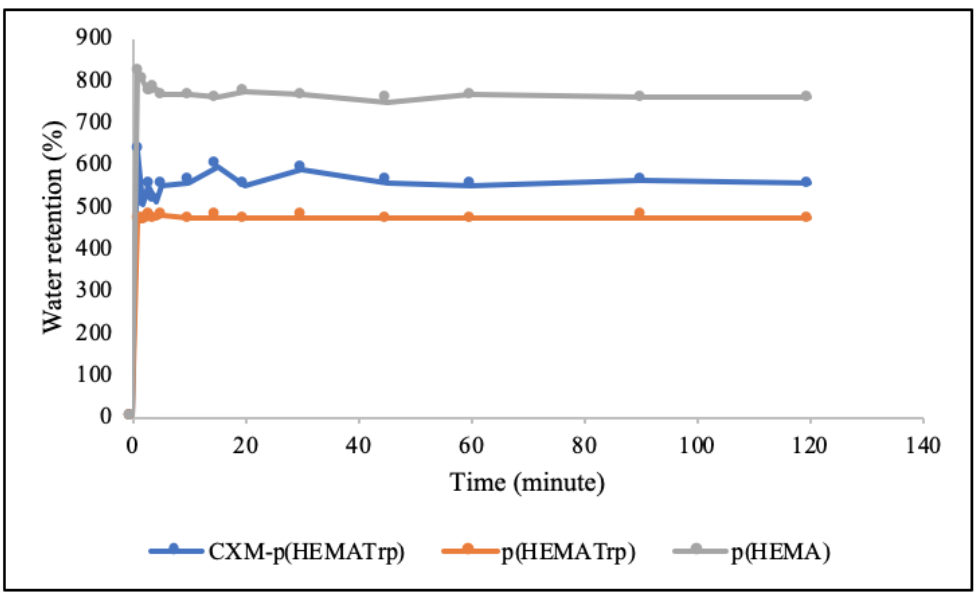

Figure 1. Water retentions of p(HEMA), p(HEMATrp) and CXM-p(HEMATrp) cryogels.

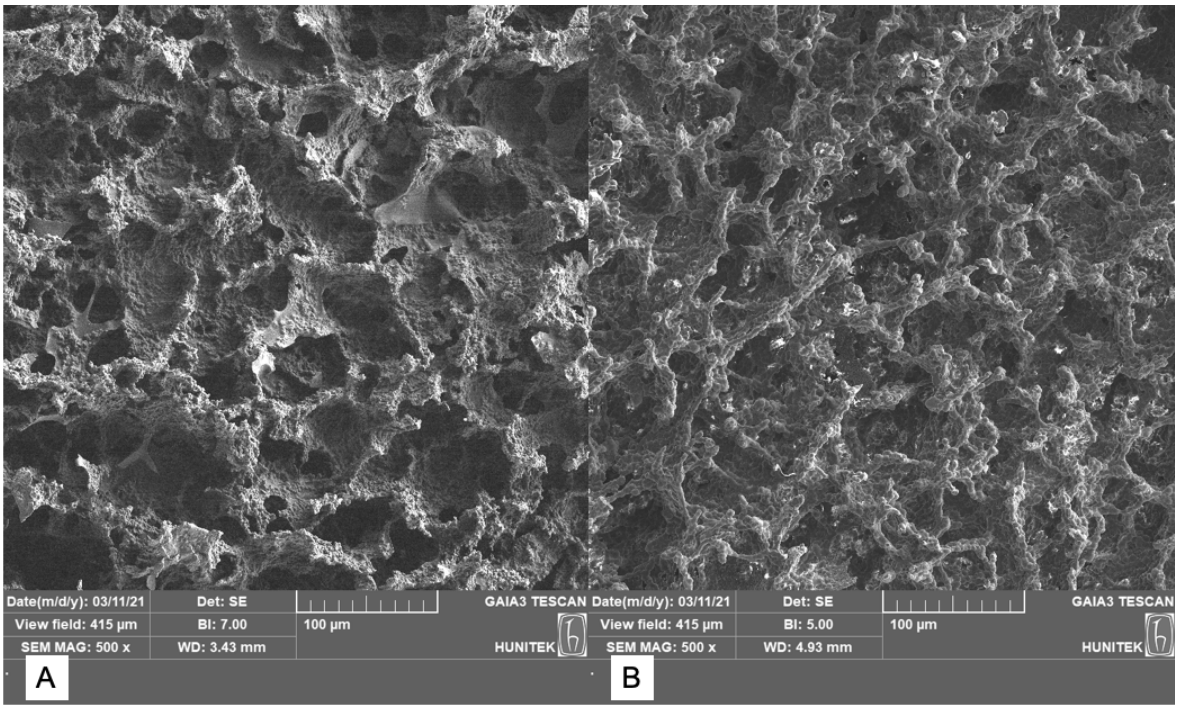

Figure 2. SEM images of (A) p(HEMATrp) and (B) CXM-p(HEMATrp) cryogels.

µm (Figure 2B), respectively. Interconnected macroporous structures of cryogels have an advantage of providing effective mass transfer.

Figure 3 shows FTIR analysis of CXM-p(HEMATrp) and p(HEMATrp) cryogels. OH- stretching bands at 3235 and 3280 cm^{-1}, CH- stretching vibrations at 2944 and 2950 cm^{-1}, 1717 and 1720 cm^{-1} C=O stretch bands at and C=C stretching bands at 1650 and 1655 cm^{-1} were obtained for CXM-p(HEMATrp) and p(HEMATrp) cryogels, respectively. NH bending at 1528 and 1531 cm^{-1}, CN aromatic bands at 1449 and 1447 cm^{-1}, amide bands at 1655 and 1650 cm^{-1} of the MATrp monomer are included in the FTIR spectra of CXM-p(HEMATrp) and p(HEMATrp) cryogels, respectively [41]. Band shifts in the FTIR spectrum of CXM-p(HEMATrp) can be attributed to 2944 cm^{-1} C-H stretching vibration, 1717 $^{-1}$ C-O stretching vibration and 1650 cm^{-1} N-H bending vibrations from CXM [42]. In comparison to p(HEMATrp) cryogels, the FTIR spectrum of CXM-p(HEMATrp) cryogel contains C-S stretching vibration at 572 cm^{-1} originating from CXM.

The drug loading capacity was determined by spectrophotometer [43] and can be identified as the concentration of loaded drug per unit weight of the polymeric material. In this study, maximum CXM loading capacities of CXM-p(HEMATrp) and p(HEMATrp) cryogels were found as 138.3 and 34.7 mg/g, respectively. The obtained results show that much greater CXM loading capacity was calculated for CXM-p(HEMATrp) than that of p(HEMATrp) cryogels. The evaluation of the functionality of non-imprinted cryogels is needed primarily for the determination of imprinting efficiency. As a consequence, selective and specific recognition regions for CXM were formed during the imprinting process.

In vitro CXM releasing profile of the CXM-p(HEMATrp) cryogels was given in Figure 4. The cumulative CXM releasing was calculated as 65.9% and 74.8% for CXM-p(HEMATrp) cryogels at the end of 6 and 120 h, respectively. It can be concluded that affinity binding cavities for CXM were successfully produced and non-specific interactions between CXM and p(HEMATrp) cryogels occurred.

Antimicrobial assay

The in-vitro antibacterial role of CXM-p(HEMATrp) cryogels was investigated against *S. aureus, E. faecalis* and *E. coli* strains. The obtained zone diameters of bacterial growth inhibition represent the antibacterial activity of CXM-p(HEMATrp) cryogels for the interested microorganisms. The results of antimicrobial assays of CXM-p(HEMATrp) and p(HEMATrp) cryogels were indicated in Figure 5. The diameters of inhibition zones of CXM-p(HEMATrp) cryogels

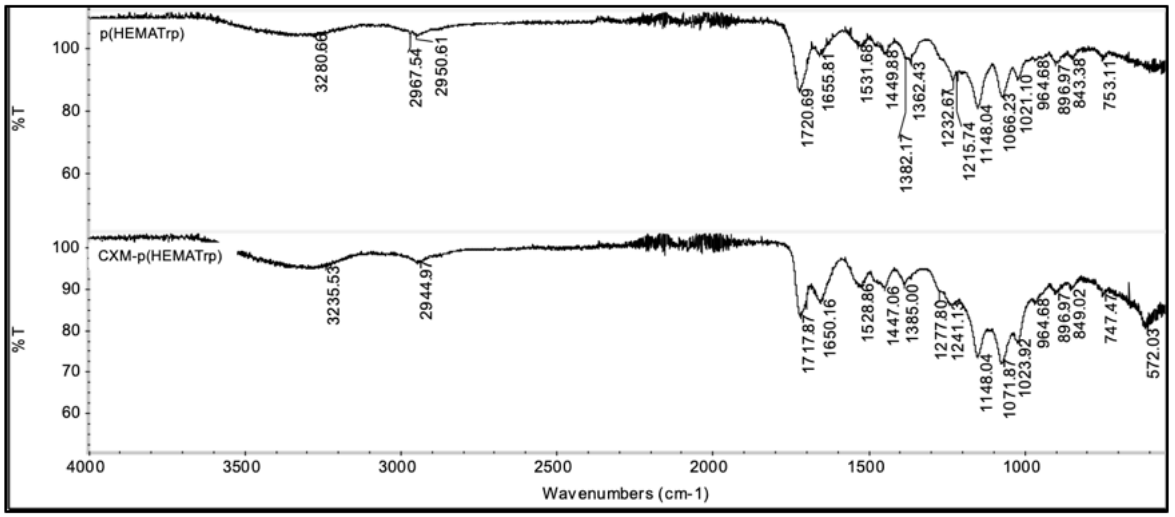

Figure 3. FTIR analysis of p(HEMATrp) and CXM-p(HEMATrp) cryogels.

for *E. coli*, *E. faecalis* and *S. aureus* were shown in Figure 5A, 5C and 5E, respectively. The zone diameters were determined as 3.1 ± 0.2, 2.9 ± 0.2 cm and 2.9 ± 0.1 for *E. coli, E. faecalis* and *S. aureus,* respectively. On the other hand, no inhibition zones around p(HEMATrp) cryogels for *E. coli, E. faecalis* and *S. aureus* were seen in Figure 5B, 5D and 5F, respectively. It means that bacterial strains grow around p(HEMATrp) cryogels for all of the tested microorganisms and p(HEMATrp) cryogels showed no antibacterial activity as estimated. It can be definitely explained that antibacterial performance of CXM-p(HEMATrp) cryogels was referred to well-designed artificial molecular recognition of CXM according to the larger inhibition zones around drug imprinted cryogels.

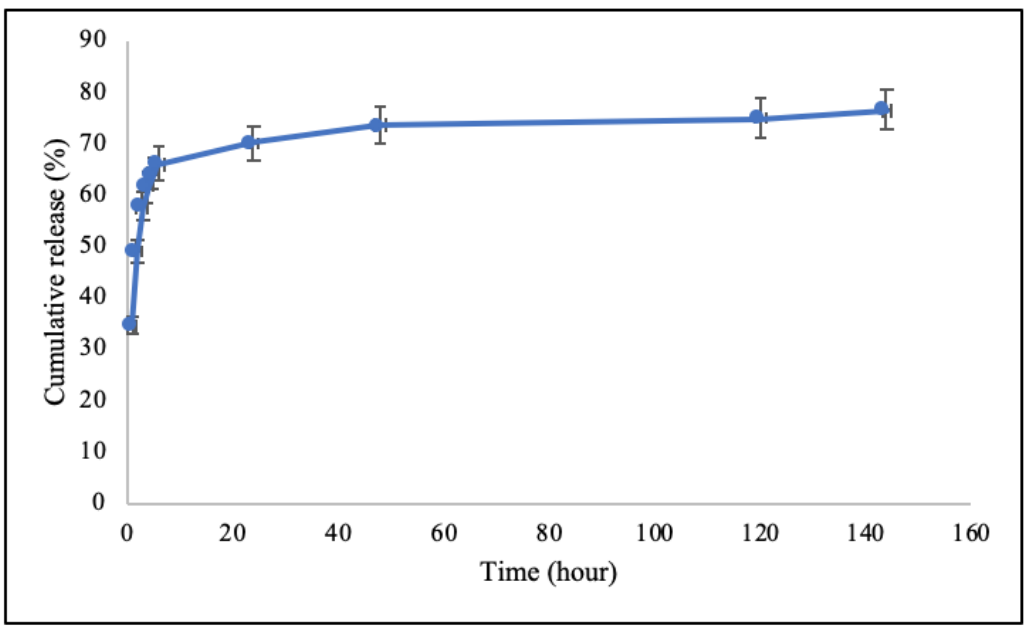

Figure 4. The cumulative release curve of CXM-p(HEMATrp) cryogels.

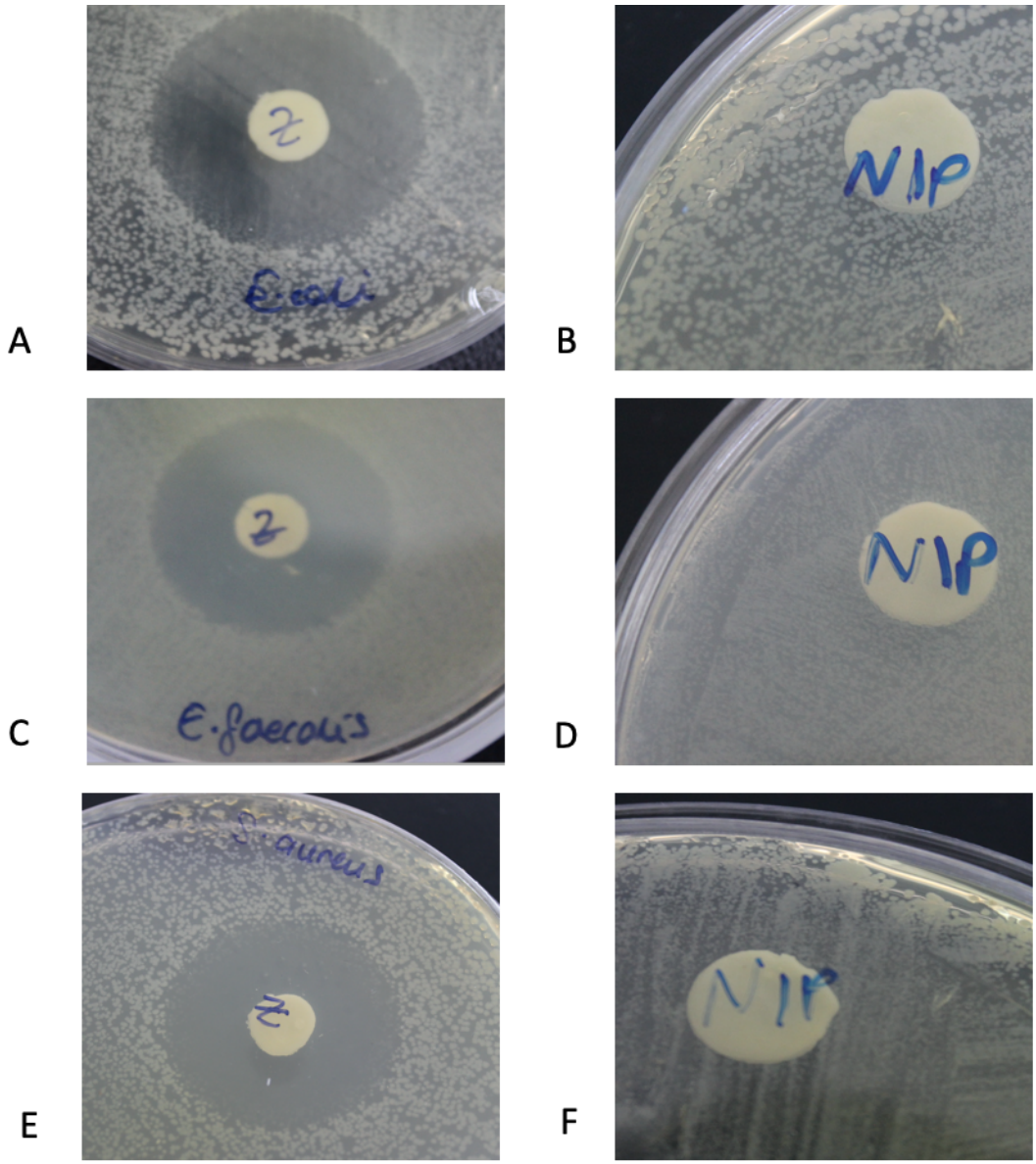

Figure 5. Antimicrobial performances of CXM-p(HEMATrp) cryogels against (A) *E. coli*, (C) *E. faecalis*, (E) *S. aureus*; Antimicrobial performances of p(HEMATrp) cryogels against (B) *E. coli*, (D) *E. faecalis*, (F) *S. aureus.*

CONCLUSION

MIPs have extensively applied to fabricate polymeric materials which have the ability to selectively capture antimicrobial agents. The other superiorities of MIPs can be listed as stability in different physicochemical conditions, providing high affinity towards target molecule, probable usage in various applications including drug release. The key advantage of proposed matrix is the usage of cryogels which are appealing type of newly generated drug release systems. Cryogels having interconnected macroporosity which provides absorption of fluidics and following, drug release. Besides, the other promising feature is predesignable manner of cryogels which enables to motivate efficient drug loading capacities for selected target drug. In the present study, it is verified that CXM loaded CXM-p(HEMATrp) cryogels can release target agent in a controlled manner. Antibacterial roles of CXM-p(HEMATrp) cryogels were proved by indicated inhibited growth of both Gram-positive and Gram-negative bacterial strains. In conclusion, molecularly imprinted materials could be potentially applied and promising systems for creating sustained-release delivery.

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