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Bakır (II) İyonu ve Histidin İçeren Polimerlerin *Saccharomyces cerevisiae* Mayası Üzerindeki Antifungal Etkileri

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ÖZ

Biyomedikal implant oklüzyonundaki ve derin yara iyileşmesindeki en ciddi endişelerden biri bakteri ve mantarların neden olduğu mikrobiyal enfeksiyonlardır. Bu nedenle, yarada, yaranın etrafında ve implantın yerleştirileceği bölgedeki enfeksiyonları önlemek veya tedavi etmek için antimikrobiyal ve antifungal özelliklere sahip malzemeler tasarlamak çok önemlidir. Kriyojel olarak adlandırılan kriyo-hidrojeller, yara iyileştirme malzemeleri için önemli bir seçenektir. Bu çalışmada, metal iyonu ve amino asit katıldığında malzemelerin antifungal etkilerindeki değişiklikleri incelemek amacıyla kriyopolimerizasyon işlemi kullanılarak çeşitli polimerler sentezlenmiştir. 2-Hidroksietil metakrilat'ın ana monomer olarak kullanılmasıyla sentezlenen polimerleri karakterize etmek için şişme testleri, Fourier dönüşümlü kızılötesi spektroskopisi, taramalı elektron mikroskobu ve X-ışını kırınımı incelemeleri yapılmıştır. Kriyojellerin antifungal aktiviteleri, fırıncı mayası veya bira mayası olarak da adlandırılan ökaryotik maya hücre modeli *S. cerevisiae* üzerinde incelenmiştir. 2-Hidroksietil metakrilat temelli polimerler gözenekli morfoloji sergilemektedir. Sonuçlar, bakır iyonlarının 2-Hidroksietil metakrilat temelli polimerlerin antifungal aktivitesinde önemli bir rol oynadığını, ek olarak histidin bağlanmasının ise hücresel metabolik aktivite yüzdelerinde artışa sebep olduğunu göstermiştir.

Antifungal Activities of Copper (II) Ion and Histidine Incorporated Polymers on Yeast *Saccharomyces cerevisiae*

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ABSTRACT

One of the most serious concerns in biomedical implant occlusion and deep wound healing is microbial infections caused by bacteria and fungi. Therefore, it is crucial to design materials with antimicrobial and antifungal properties to prevent or cure infections in the wound, its surroundings, and the site where the implant will be placed. Cryo-hydrogels, called cryogels, are a valuable option for wound healing materials. In this study, various polymers were synthesized using the cryopolymerization process in order to examine the changes in the antifungal effects of the materials when metal ion and amino acid are incorporated. Swelling tests, Fourier transform infrared spectroscopy, scanning electron microscopy, and X-ray diffraction investigations were performed to characterize the polymers synthesized using 2-hydroxyethyl methacrylate (HEMA) as main monomer. The antifungal actions of the cryogels were examined on the eukaryotic yeast cell model *S. cerevisiae*, also referred to as baker's yeast or brewer's yeast. HEMA-based polymers exhibit porous morphology. Results showed that copper ions play an essential role in the antifungal activity of the HEMA-based polymers while attachment of additional histidine causes the recovery of cell metabolic activity.

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INTRODUCTION

Hydrogels are water-insoluble gels having porous polymer networks with high liquid absorption due to physical and/or chemical cross-links [1]. In addition to having exceptional swelling characteristics, hydrogels are also biocompatible, biodegradable, and environmentally friendly materials [2], [3]. They can frequently be assessed in the category of materials for biomedical uses since they also exhibit tunable physical and chemical characteristics that mimic the extracellular matrix (ECM) in tissues [4], [5]. Although hydrogels have a wide range of uses due to their properties, they also have drawbacks. For example, hydrogels are very soft and brittle when swollen with water, so their mechanical strength is not stable [6]. On the other hand, the pore size of hydrogels, mainly when used as a tissue scaffold, causes problems in intercellular nutrient transport and waste removal, and cell adhesion, which becomes complicated and causes an irregular distribution of cells in the gel [7]. In recent years, cryogels, which belong to the class of hydrogels, have begun to be produced via gelation at temperatures below the freezing point of water, which is called cryogelation [8]. The melting of these ice crystals under room temperature conditions creates interconnected macroporous networks [9]. Especially against the limiting properties of small-pore hydrogels, macroporous cryogels present some privileges such as effective mass transport of macromolecules, much mechanical stability, and high micro-environmental biocompatibility thanks to the cryogelation method [10]. Thus, there is an increasing interest in using cryogels since having advantages over traditional type hydrogels [11]. Cryogels are mainly applied in biotechnological fields due to their fast mass flow properties supported by the elastic morphological structures combined with mechanical and chemical stability [12]. For biotechnological approaches, cryogels can be considered, such as drug delivery systems [13], 3D scaffolds for cell cultivation [14], tissue engineering [15], also bioseparation as chromatographic materials for the separation and purification of biological macromolecules [16]. Recently, sensor- and impedance-based studies of cryogels have been popular for bioelectronic applications [17]–[19].

Some of fungi can act as pathogens and disruptive effect the immune system. Therefore, it is thought that materials with antifungal properties can provide an essential deterrent against fungal infections [20]. Recently, wound dressing materials exhibiting antifungal and antibacterial effects have become widespread [21]–[23]. They should have some significant properties such as easy and low-cost production, reproducibility, non-toxic, antimicrobial/ antifungal and so on [24]. Various synthetic or natural polymer-based wound dressing materials have been produced in the literature, the main ones are films [25], topical agents [26], transdermal patches [27], and polymers including nanomaterials [28] and hydrogels [29]. On the other hand, though some of these materials prevent bacterial infections of skin wounds, they may face various limitations due to the high production cost, poor mechanical stability, inability to remove the lesion without damaging it, or not creating a moist environment [30]. So, among these materials, cryogels could be one of the best options since their macroporous structure, and favorable swelling ratios provide a moist environment that is beneficial for wound healing while also allowing oxygen permeability and exudate drainage [31]. Also, compared to conventional dressing materials such as cotton, wool, or gauze, they facilitate their spontaneous exfoliation and degradation, avoiding the discomfort and secondary trauma caused by changing dressings [32], [33].

2-Hydroxyethyl methacrylate (HEMA) is commonly used to synthesize cryogels as the main monomer since its good biocompatibility via its hydrophilic character and tunable mechanical properties [34], [35]. Hence, poly(HEMA) (PHEMA) based hydrogels and cryogels are getting more popular from they were used to produce contact lenses [36] to today's applications related to biomedical and biochemistry fields, including drug delivery devices [13], wound dressing materials [37], electroconductive materials [38], and scaffolds [39]. In this paper, HEMA-based cryogels including PHEMA (H1), PHEMA-NVP (H2), PHEMA-NVP-Cu(II) (H3), PHEMA-NVP-Cu(II)-His (H4), and PHEMA-NVP-His (H5) cryogels were synthesized via cryopolymerization. They were characterized by swelling and porosity tests, Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and X-ray diffraction (XRD) studies. Finally, their antifungal activities were investigated against four different strains of *Saccharomyces cerevisiae* yeast. According to the best of our knowledge, this is

the first study which these Cu(II) and/or His containing cryogels were synthesized and investigated for their possible antifungal activities on the opportunistic yeast *S. cerevisiae*.

MATERIALS AND METHODS

All reagents used for the preparation of cryogels namely HEMA, N-vinyl pyrrolidone (NVP), N, N′-methylene bis(acrylamide) (MBA), ammonium persulfate (APS), L-histidine, N, N, N', N'-tetramethyl ethylene diamine (TEMED) and copper (II) nitrate hydrate were obtained from (Sigma-Aldrich, St. Louis, Missouri, USA).

The synthesis of the poly(HEMA-NVP) cryogel has been described in an earlier study [34]. Five different cryogels denoted as H1, H2, H3, H4 and H5 were produced as follows: First, MBA (240 mg) was dissolved in 12.0 mL of deionized water (DW), and then HEMA (1.3 mL) and NVP (10.7 µL) were added to the solution. $Cu(NO_3)_2{\cdot}3H_2O$ (4.8 mg) for H3, $Cu(NO_3)_2{\cdot}3H_2O$ (4.8 mg) and histidine (15.5 mg) for H4 and histidine (15.5 mg) for H5 cryogels were also added to the solutions. After adding APS and TEMED, the solutions were transferred to the syringes placed in the ice box and polymerization was carried out at -18 °C for 24 h. Then, cryogels were thawed at room temperature (RT) and subjected to DW to remove unreacted monomers and other residues from the polymeric structures.

The characteristic functional groups of HEMA-based cryogels were obtained using an attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR; Spectrum OneTM, Perkin Elmer, Massachusetts, USA). The surface and bulk structure of cryogels were examined with a scanning electron microscope (SEM) (Quanta FEG 250, FEI, USA). X-ray diffraction (XRD) analysis was carried out with a powder X-ray diffractometer (PANanalytical, Empyrean model, UK) to determine the crystalline structures of the cryogels. For this purpose, each of the powdered samples was performed based on scanning degree $0° \leq 2\theta° \leq 90°$ and scanning speed 1°/min.

The initial weights of the dry cryogels were measured and recorded. These cryogels were then placed in a beaker containing distilled water and swelled for 2 h. Measurements made by taking two samples were repeated three times at different times. Swelling degree and porosity of the cryogels were calculated using the formulas below:

$$Swelling\ degree\ (\%) = \frac{W_s - W_d}{W_d} \times 100 \quad (1)$$

$$Porosity\ (\%) = \frac{w_s - w_d}{w_s - w_{sq}} \times 100 \quad (2)$$

Here; W_d is the weight of dried cryogels (g) while *Ws* and *Wsq* are the weights of the cryogels after swelling (g) and squeezed (g), respectively [40].

The possible antifungal effects of the HEMA-based cryogels (H1-H5) were evaluated against four different strains of yeast cells including wild type *S. cerevisiae* BY4741 and its isogenic cytosolic catalase T (CTT1), thioredoxin-disulfide reductase (TRR2) and glutathione synthase (GSH2) deletion mutants. The genomic background of the wild type strain is MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0. Both the wild and the mutant strains constructed by kanMX4 cassette deletion of the specified genes were obtained from Dharmacon (Lafayette, CO). We have used the deletion mutants in order to evaluate the possible protective roles of these important antioxidative enzymes against the Cu(II) and/or His containing HEMA-based cryogels. The routine maintenance of all the strains was carried out at 30 °C for 2 days on agar plates prepared with YPDA medium (pH: 5.6) containing 10 g yeast extract, 20 g peptone, 20 g glucose and 20 g bactoagar per liter. the cells were growth until their mid-exponential phase in YPD medium without bactoagar under same conditions for the cytotoxicity assay. 200 mg/L geneticin was also added to the medium of the mutant strains for the selective growth. Then, cryogels were treated with these cells at an initial concentration of 5 x 10^7 CFU/mL for 24 h. After incubation period, the mediums were removed and the remaining gels were washed twice with the phosphate-buffered saline (PBS), pH 7.4. The metabolic activity of the cells was determined by MTT assay in the washing solution. Briefly, precipitated cells from the washing solutions were mixed with 50 µL of 0.5 mg/mL MTT solution and the incubation was carried out for 2 h at 37 °C. Then, all the samples were mixed with 500 µL of propan-2-ol which contains 0.04 M HCl, and the mixtures were

vigorously vortexed in order to remove MTT-formazan from the cells. After centrifugation at 10 000 x g for 2 min., the absorbance values of the clear supernatants were measured against controls at 570 nm [41]. The results were expressed as metabolic activity percentages in comparison with untreated control which was accepted as 100%.

All the experiments were carried out in triplicate and the data are reported as the mean of percentage of cell metabolic activity ± S.E.M. A one-way analysis of variance (ANOVA) test was utilized via GraphPad Prism version 6.01 (GraphPad Software Inc.; La Jolla, CA) to statistically assess the variance differences.

RESULTS AND DISCUSSION

Figure 1 shows FT-IR spectra of both P(HEMA) and P(HEMA-NVP) cryogels. The O-H groups were responsible for a broad band in the spectra around 3300 cm^{-1}. The peaks of the ester carbonyl groups of P(HEMA) and P(HEMA-NVP) cryogels were shown at 1715.97 and 1720.72 cm^{-1}, respectively. The peaks around 1450 cm^{-1} and 1150 cm^{-1} were attributed to the C–N stretching and C–O–C bending bands, respectively [42]. The peaks of NVP were not as pronounced as there was around 100 times more HEMA in the reaction mixture than NVP. The carbonyl stretching banding of NVP, however, might have contributed to the peak's increased intensity at 1652 cm^{-1} [43], [44].

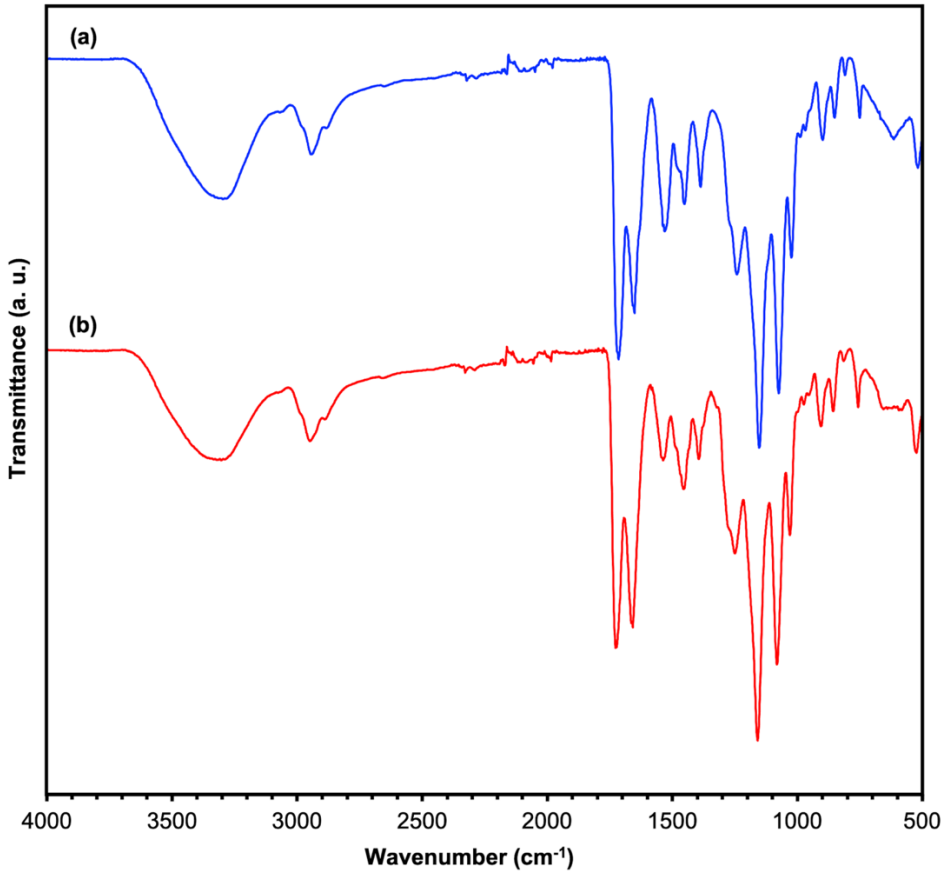

Figure 1. *FTIR spectra of (a) P(HEMA) and (b) P(HEMA-NVP) cryogels*

Figure 2 presents the optical images of the cryogels in the swollen state with DW. All of the cryogels were opaque, allowing light from various domains to be scattered to be seen [45]. Surface morphologies of the PHEMA and P(HEMA-NVP), i.e., H1 and H2, respectively, cryogels are presented in Figure 3. The cryogels exhibited interconnected macro and micro-porous structures and a network topology. According to data given in Table 1, all of the cryogels exhibited high swelling values and porosities.

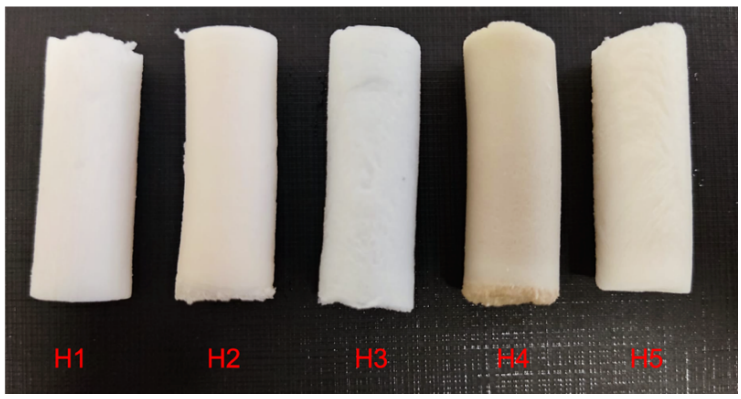

Figure 2. *Optical images of the cryogels.*

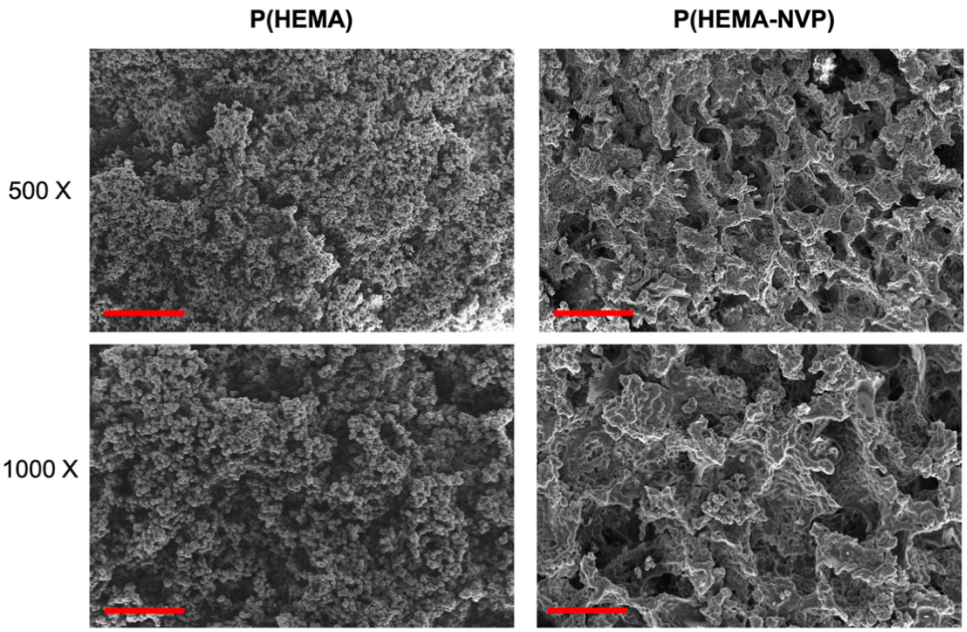

Figure 3. *SEM images of P(HEMA) and P(HEMA-NVP) cryogels. Scale bar: 100 µm for × 500; and 50 µm for × 1000.*

Table 1. *The swelling and porosity values of the cryogels*

Cryogel	Swelling (%)	Porosity (%)
H1	586 ± 51	147.34 ± 3.2
H2	590 ± 62	122.91 ± 4.6
H3	565 ± 48	118.36 ± 2.7
H4	584 ± 54	108.51 ± 3.4
H5	565 ± 36	121.92 ± 4.2

For the confirmation of copper in the cryogels, powder XRD analysis of both P(HEMA-NVP) [H2] and P(HEMA-NVP)-Cu(II) [H3] were utilized, as presented in Figure 4. While there was no specific peak in the XRD image of H2, the XRD pattern of H3 exhibited a peak at 45.71° confirming to copper incorporation into the cryogel matrix [46].

The antifungal activities of the cryogels were investigated on eukaryotic yeast *S. cerevisiae* which is also known as baker's yeast or brewer's yeast. This organism is the first eukaryotic model whose genome was fully sequenced. *S. cerevisiae* differs from other microorganisms in that it is susceptible to genetic manipulation and has similar biochemical pathways with higher eukaryotic organism [47]. In addition, this yeast is of critical importance

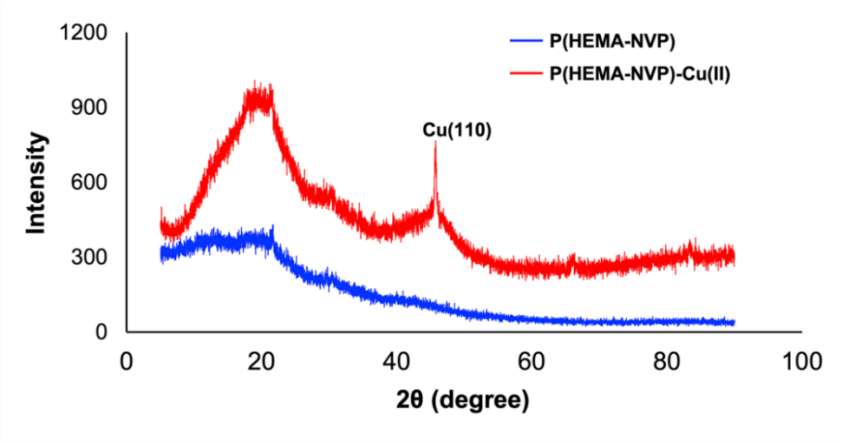

Figure 4. *XRD images of the P(HEMA-NVP) and P(HEMA-NVP)-Cu(II) cryogels.*

in the production of many food and alcoholic beverages [48]. On the other hand, it can form durable colonies in many different natural environments [49]. For instance, it causes spoilage in foodstuffs in terms of their physical and sensory properties as a result of yeast metabolic activity. In addition, studies have shown that this strain can have negative effects on human health and care should be taken when using *S. cerevisiae* probiotics especially in immunosuppressed or critically ill patients [50]–[52]. Because of all these reasons, we have investigated the possible antifungal activities of the cryogels by using *S. cerevisiae* strains. It is widely seen in the literature that this yeast is used to determine the antifungal activities of various materials [53]–[55].

The metabolic activity percentages after treatment with H1-H5 cryogels of four different yeast strains precipitated from washing solutions were presented in Figure 5. According to the results, there was no significant difference between H1 and H2 groups and all the strains showed same cell metabolic activity trend for the remaining groups. H3 was found as the most antifungal cryogel for both wild and mutant yeast strains. The challenge with H3 decreased metabolic activity percentages to 47.54±1.53, 47.42±1.99, 29.17±1.06 and 49.77±1.53 in wild, ΔCTT1, ΔTRR2 and ΔGSH2 strains, respectively. From these data, it is clear that ΔTRR2 mutant was the most sensitive strain to the treatment. These cells were also found more sensitive compared to other ones to the rest of applications. After treatment with H4 and H5 gels, the metabolic activity percentages were determined as 46.73±1.97 and 52.17±1.19, respectively, between which there was no significant difference unlike other strains. From the findings, it can be interpreted that copper ions are essential for the antifungal activities of these HEMA-based cryogels, and additional histidine attachment generally leads to the recovery of cell metabolic activity.

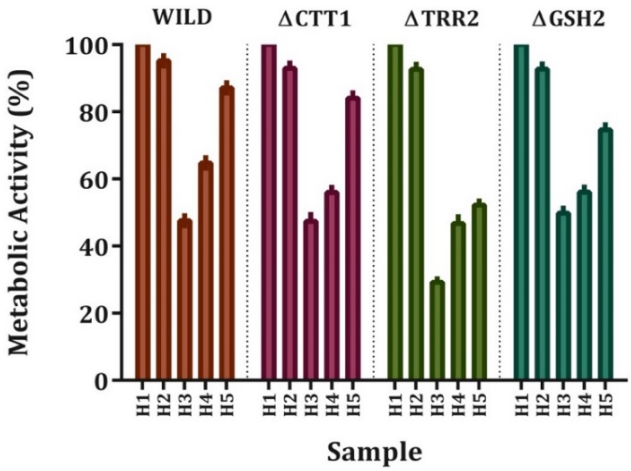

Figure 5. *The effects of cryogels treatment for 24 h on cell metabolic activities of Saccharomyces cerevisiae strains. Cell metabolic activity percentages calculated in comparison to the relevant control group were used to display the results. The mean and ± SEM of three separate trials is displayed in data with error bars.*

CONCLUSIONS

In biomedical engineering, it is important to design materials with antimicrobial and antifungal activities to stop or cure infections around wound area and implant site. In this work, the cryopolymerization method was used to create compressible, spongy HEMA-based polymers. The monomer NVP was selected to form a metal-chelate complex with Cu(II) ions and histidine. The antifungal activities of polymers were investigated on the eukaryotic yeast cell model *S. cerevisiae* and the results indicate that copper ions are necessary for the antifungal action of the HEMA-based polymers, and further histidine attachment often results in the restoration of cell metabolic function.

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