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Investigation of the antifungal activities of cryogels for potential use as wound dressing materials

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ABSTRACT: The fungus can act as a pathogen and disrupt the immune system. Therefore, materials with antifungal properties can provide an essential deterrent against fungal infections. The eukaryotic yeast cell model *Saccharomyces cerevisiae*, known as baker's yeast , is widely used in food industry in baking, winemaking, and brewing. Moreover it is also commonly used as a probiotic for treating gastroenteritis and regulating the endogenous flora and immune system. However, in recent years, there has been an increase in cases of fungemia caused by *S. cerevisiae* and its subspecies, *S. boulardii*, and and therefore caution should be exercised when using it as probiotic especially in immunocompromised individuals or low weight infants. Cryo-hydrogels, called cryogels, are mainly used in biotechnological fields due to their fast mass flow properties supported by the elastic morphological structures combined with mechanical and chemical stability. Also, these polymers are a valuable option for wound-healing materials. In this study, HEMA-based cryogels containing different amounts of N-Vinylformamide were prepared and characterized to investigate their anti-fungal activities on *Saccharomyces cerevisiae*. The results indicated that, PHEMA/PNVF cryogels showed considerable effectiveness against this opportunistic strain. So, PHEMA/PNVF can be considered a potential wound dressing material for future studies

Keywords: Cryogels, fungemia, anti-fungal activity, *Saccharomyces cerevisiae*.

INTRODUCTION

Hydrogels are cross-linked, three-dimensional network polymers that swell without dissolving in water. Hydrogels show remarkable similarities to living tissues due to many physical properties, such as having plenty of water in their structures, biocompatibility, and being soft and flexible (Manzoor et al., 2022). Cryogels can be called cryo-hydrogels, a member of a gels family, synthesized at temperatures below the solvent's freezing point (Lozinsky, 2018). Cryogels, as compared to traditional hydrogels, have large pore sizes, more swelling ratios, a short diffusion path, good biocompatibility, also high mechanical and physical stability (Su and Okay, 2019). So, in recent years, cryogels have been preferred over hydrogels (Karacan and Okay, 2013). Cryogels can be prepared in different shapes (cylindrical, sphere, etc.), sizes (macro- or micro-), or functions such as columns or membranes (Çetin and Denizli 2019). Cryogels have a wide range of applications covering the fields of insulation, environment (Şarkaya et al., 2018), food (Coria-Hernández et al., 2018), health (Shih et al., 2018), electronics (Sevgül Bakay et al., 2022), and drug biotechnology (Ferreira et al., 2019).

The fungus has a disruptive impact on the immune system and can act as an infection. It is believed that substances having antifungal characteristics can act as a crucial deterrent against fungus infections (Zumbuehl et al., 2007). Developing new materials with antimicrobial activity has become necessary with the prevalence of microbial infections as diseases impaired wound healing and biomedical implant failure. Also, there is an increasing need for materials that can resist these negativities and have antifungal properties (Ailincai et al., 2016). In recent years, materials for wound dressing have been developed to combat pathogens' growing resistance, which can result in serious infections, challenging wound healing, and the loss of many lives (Zhao et al., 2017). Hydrogels, a class of highly hydrated biomaterials produced naturally or synthetically, can be a helpful starting point for designing antimicrobial materials (Veiga and Schneider, 2013). Before considering hydrogels as a wound dressing material for fungi infections, we aim to investigate the antifungal activities of cryogels in this study. For this purpose, we prepared 2-Hydroxyethyl Methacrylate (HEMA)-based cryogels. PHEMA cryogels are highly hydrophilic with a high swelling degree, biocompatible, hydrolytic, and chemically stable. From this point of view, PHEMA can be used as a tissue scaffold in medicine and controlled drug release systems (Roointan et al. 2018), soft contact lenses (García-Millán et al., 2015), and tissue engineering (Dragusin et al., 2012). PHEMA-based materials are tolerant of embedded cells (García-Uriostegui et al., 2018). Also, PHEMA cryogel is hardly self-degrading because of its high biostability (Moghadam and Pioletti, 2016).

Saccharomyces cerevisiae, commonly referred to as baker's yeast and used in the bread sector, is also employed as a probiotic for treating gastroenteritis and controlling the immune system and endogenous flora (Nash et al., 2017). Although it is a rare source of infection in people and is a member of the human fungal microbiome, the usage of it as probiotics in immunocompromised people have recently increased,the incidences of fungemia caused by this strain. It is noted that it should

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only be used cautiously in sick patients and newborns who are underweight (McFarland, 2010). Additionally, since the critical study by Munoz et al., it has been accepted that *S. cerevisiae* fungemia may occur after using probiotics containing *S. boulardii*. In their work, it was convincingly shown that strains isolated from a patient's blood and strains isolated from the probiotic preparation were genetically identical; this is an observation that supports the hypothesis of translocation from the gut to the bloodstream (Muñoz et al., 2005).

In this study, PHEMA cryogels were selected as main monomer based on their high degree of hydrophilicity, biocompatibility, and cell-trap tolerance (García-Uriostegui et al., 2018). N-Vinylformamide (NVF), on the other hand, was utilized to produce a copolymerization reaction with HEMA. One of the most popular monomers, acrylamide, has an isomer called NVF. NVF has stronger reactivity than acrylamide and is water soluble, giving it a wider range of applications thanks to its low toxicity (Shi et al., 2008; Luo et al., 2021). As a result, polymers including NVF can be employed in many of the same applications as acyrlamides (Suekama et al. 2013). In this study, PHEMA/PNVF cryogels were synthesized, characterized and the anti-fungal activities of these cryogels were investigated against wild type yeast strain of *Saccharomyces cerevisiae*. The results showed that these PHEMA/PNVF cryogels have significant antifungal effects and should be evaluated for their further applications as antifungal materials.

MATERIALS AND METHODS

2-Hydroxyethyl methacrylate (HEMA) , N-Vinyl formamide (NVF), N,N'-methylene bisacrylamide (MBAAm), Tetramethylethylenediamine (TEMED) and Ammonium persulfate (APS) were purchased from Sigma (Sigma Chemical Co., USA). All chemical reagents were of analytical purity. Ultra distilled water was used in the experiments.

Preparation of cryogels

Our previous work has precisely stated the procedure for synthesizing cryogels (Şarkaya and Allı 2021a; Şarkaya et al. 2022). According to this, 1.3 mL of HEMA was dissolved in 5 mL water, and 0.2 g of MBAAm was dissolved in 10 mL water. The two solutions are then mixed. 20 mg APS was added to the mixture and thoroughly dissolved. Solutions containing varying amounts of N-Vinylformamide (i.e., 22 and 44 µL of N-Vinylformamide, abbreviated PHEMA/PNVF22 and PHEMA/PNVF44) prepared, then, 25 µL of TEMED was added, and the solution was dispensed into 5 mL plastic syringes after mixing (0.8 cm in diameter). Cryogel solutions were cooled to -18 °C and held there for 24 hours. After 24 h, the cryogel produced by free-radical polymerization was removed from the freezer. The cryogel was washed with distilled water for several hours to remove unreacted monomers. The same procedure described above synthesizes bare PHEMA cryogel without adding NVF monomer to ensure comparable results.

Characterization of cryogels

For the gelation efficiency (G) of the cryogels, the swollen cryogel sample was dried in a lyophilizer. After reaching the constant weight, the mass of the cryogel sample was determined, and the gelation efficiency was calculated using the following equation (Eq) (1):

$$G = \frac{W_D}{W_t} \times 100 \qquad \text{(Eq) (1)}$$

According to equality, w_D is the dry weight of the cryogel, and w_t is the total mass of monomers in the polymerization mixture.

The dried cryogels (W_D, g) were soaked in deionized water at room temperature for 2 hours and roughly wiped with damp filter paper and weighed (w_1, g). The cryogels were then subjected to mechanical compression to remove the water in the macropores and weighed again (w_2, g). Then, the swelling degree (S.D.), swelling ratio (S.R.) and macroporosity (M) of the cryogels were calculated using the following equations (Eq) (2), (Eq) (3) and (Eq) (4), respectively:

$$S.D. = \frac{W_1}{W_D} \qquad \text{(Eq) (2)}$$

$$S.R. = \frac{W_1 - W_D}{W_D} \times 100 \qquad \text{(Eq) (3)}$$

$$M(\%) = \frac{W_1 - W_2}{W_2} \times 100 \qquad \text{(Eq) (4)}$$

Dried cryogels were placed on the ATR (Nicolet iS10 ATR-FTIR, Thermo Scientific, Madison, WI, USA) probe and then FTIR spectra were obtained. The surface and pore morphology of cryogels scanning electron were analyzed with a microscope (SEM) (ZEISS GeminiSEM 500). Dried cryogels were coated with a molecular gold-palladium mixture on the SEM sample holder for 2 minutes, and then SEM images of the cryogels were obtained at various magnifications.

Determination of Yeast Cell Viability

The antifungal effects of the polymeric cryogels PHEMA/PNVF/22 and PHEMA/PNVF/44 were evaluated against wild type yeast strain of Saccharomyces cerevisiae BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0). The PHEMA cryogel was used as control. The routine maintenance of the yeast cells was carried out on agar plates prepared with YPDA medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L bactoagar, pH: 5.6) at 30 °C for 2 days. For the cytotoxicity assay, the cells were growth in YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose, pH: 5.6) at 30 °C until their early-exponential phase (OD660nm~0.5). Then, the cells were treated with the 0.15 gr of polymers at an initial concentration of 1x107 CFU/mL for 24 h. After incubation period, the mediums were removed and the polymers were washed with the phosphate-buffered saline (PBS) with 150 rpm agitation for 1 h. The cell viability of the cells released to the washing solution was determined by Colony Forming Unit (CFU) assay. In the assay, 1 mL from the samples was taken and serially diluted in PBS. After the diluted samples were incubated at 30°C for 2 days, the appeared yeast colonies were counted to determine the percentage of CFU on PHEMA/PNVF/22 and PHEMA/PNVF/44 agar plates with respect to that on PHEMA control agar.

All the experiments were carried out in triplicate and the data are reported as the mean percentage of cell viability ± S.E.M. The differences in variance were analyzed statistically using a one-way analysis of variance (ANOVA) test by GraphPad Prism version 6.01 (GraphPad Software Inc.; La, Jolla, CA).

RESULTS AND DISCUSSION

Polymer analysis

The synthesized cryogels for this study (shown in Figure 1) have a spongy-elastic structure, an opaque appearance, and a cylindrical shape. PHEMA cryogels have an inter-network-like porous structure due to their crosslinking with MBAAm. This way, cryogels can uptake much water in this reticulated structure. The water in the pores of the polymer can be easily removed after any mechanical compression of the cryogel. On the other hand, when the dehydrated compressed cryogel piece is left to swell in the water again, it regains its original size within seconds. In addition, despite incorporating different concentrations of NVF monomer into PHEMA cryogels, no visible difference was detected in the optical images of the cryogels.

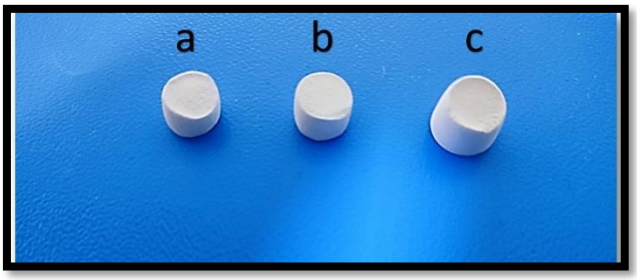

Figure 1. *Optical images of cryogels: a) PHEMA, b) PHEMA/PNVF/22 and c) PHEMA/PNVF/44*

The swelling behaviors of PHEMA, PHEMA/PNVF22, and PHEMA/PNVF/44 cryogels in water were investigated, and the results were given in Table 1. Accordingly, the swelling behavior of the PHEMA cryogels increased numerically with the concentration of the NVF monomer, which was added to the PHEMA structure as a copolymer. This can be exbareed by the hydrophilic nature of the NVF monomer, as well as by the increase in the number of pores that water can be absorbed in the polymer. On the other hand, the pore content and distribution of cryogels directly affect the activities in cell studies. For this reason, it is an easy-to-apply and fast-resulting method that can give an idea for the adhesion and proliferation of the cells of the cryogel, whose macroporosity is prepared. This study shows that the macroporosity of PHEMA cryogels containing NVF is higher than that of bare PHEMA cryogel, as can be seen from the macroporosity grade results in Table 1.

Table 1. *Results for the swelling behaviour of cryogels*

Cryogels	S.D.	S.R. (%)	M (%)	G
PHEMA	5.94	494.0	71.82	91.1
PHEMA/PNVF/22	6.11	511.72	71.04	92.1
PHEMA/PNVF/44	6.71	571.55	72.62	92.3

The infrared spectra of the PHEMA-based cryogels were shown in Figure 2. For PHEMA cryogel, the characteristic bands for PHEMA were observed at 3348 cm^{-1} (O-H stretching), 1709 cm^{-1} (C=O stretching), 1245.52 and 850.14 cm^{-1} (C-O and C-O-C stretching bands, respectively). Also (C-H) stretching bands were observed at 2948.75 cm^{-1}. These results are also compatible with previous literature studies (Bayrak et al. 2021). NVF, as a comonomer of HEMA-based cryogel (PHEMA/PNVF/44), was confirmed by the presence of the peak observed around 1244.58 cm^{-1} belonging to the C-N vibrations. The peaks at 1651 cm^{-1} and 1533 cm^{-1} indicated the Amide I and Amide II bonds, respectively. Given the proximity of the -OH and NH- peaks in FTIR spectra, it was assumed that the peak observed around 3287.86 cm^{-1} covered these two bands (Şarkaya and Allı, 2021a; Şarkaya et al., 2022).

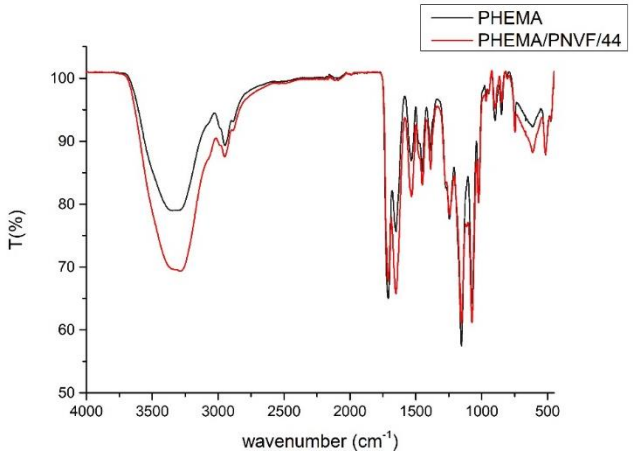

Figure 2. *FTIR spectra of PHEMA and PHEMA/PNVF/44 cryogels*

The porous morphological structure of the cryogels was investigated using field emission scanning electron microscopy (FE-SEM) in Figure 3. According to the images, there was no significant difference between the morphologies of the cryogels. Although all cryogels have highly porous structures, it was seen that the pores are not homogeneously distributed. This macroporous structure of cryogels is due to ice crystals. As a result of the melting of the ice crystals formed after the freezing reaction of the water molecules used as the solvent in the structure of the cryogels during the polymerization, a macroporous system associated with each other is formed. Cryogels' porous structure is important for cell attachment and the transport of residues or other specific species.

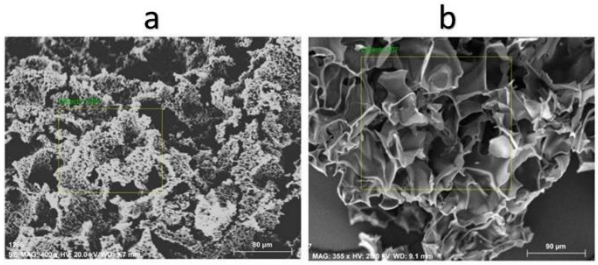

Figure 3. *SEM images of PHEMA and PHEMA/PNVF44 cryogels*

Antifungal Effects of PHEMA/PNVF Cryogels

In previous studies (Şarkaya and Allı, 2021b; Şarkaya et al., 2022), the characterizations of the cryogels were comprehensively studied. In addition, cytotoxicity tests of HEMA-based cryogels were also examined, and it was reported that they showed biocompatibility (Şarkaya et al. 2022). These results motivate us to investigate microbial activities of these cryogels. So, antifungal activites of PHEMA/PNVF cryogels are investigated against *S. cerevisiae* in this study.

The antifungal effects of PHEMA/PNVF polymers against *S. cerevisiae* yeast were presented at Figure 4. As can be seen from the results, PHEMA/PNVF polymers containing NVF in varying concentrations statistically significantly decreased yeast cell viability percentages compared to control group. PHEMA/PNVF/22 and PHEMA/PNVF/44 polymers effectively decrease cell proliferation to about %32 and 18, respectively. In current literature, there are some findings which shows the

antiproliferative activity of PNVF and its derivative poly(vinyl amine) (PVAm) against the pathogenic fungi Candida albicans ((Shandil et al., 2017; Sütekin et al., 2021; Demirci et al., 2022)). The main common result of these studies is that while PNVF shows moderate antifungal action, PVAm obtained from its acidic hydrolysis demonstrated much more potent antimicrobial activity against this pathogen. However, we have found that PNVF is quite effective on budding yeast *S. cerevisiae*. Although this yeast is not a pathogenic organism, it is accepted as an opportunistic species.

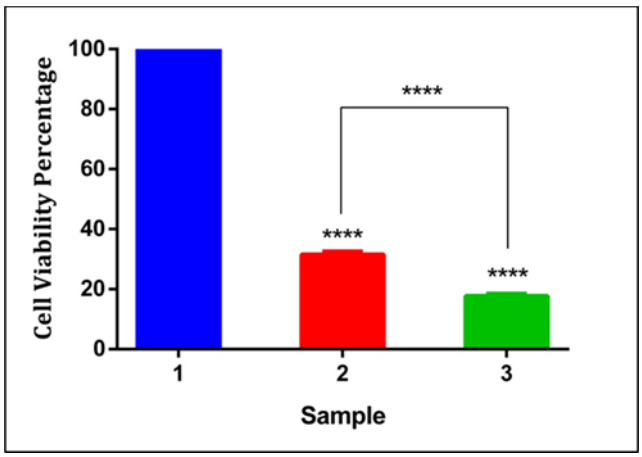

Figure 4. *The viability percentages determined by CFU assay in yeast cells treated with PHEMA/PNVF/22 (2) and PHEMA/PNVF/44 (3) compared to the PHEMA control (1). Data with error bars show the mean ± S.E.M of three experiments. ****=p<0.0001 denote significant differences between control and other studied group or indicated groups by Tukey's multiple range tests.*

Fungemia and other severe infections in the various systems of the body particularly in immunosuppressed or critically ill patients are the main issues with this organism (Muñoz et al., 2005; Dynowska et al., 2006; Algazaq et al., 2017). On the other hand, in a current study the skin infection caused by S. cerevisiae was described in an elderly Additionally, the skin infection caused by S. cerevisiae was currently described in an elderly but immunocompetent person (Belmourida et al., 2021). So when our data are evaluated in the light of literature findings it would not be wrong to say that the antifungal effect of PNVF varies depending on the species. Although further studies are needed to elucidate its mechanism of action, its potential to be used as a wound-healing antifungal biomaterial should not be ignored.

CONCLUSION

In this study, bare PHEMA and PHEMA/PNVF cryogel series was prepared and characterized. The main goal of this study was to comparatively investigate of the antifungal activities of PHEMA/PNVF cryogels against *S. ceravisiea*. When the antifungal activities of these cryogels, which are known to be biocompatible, were evaluated, it was determined that PHEMA-NVF cryogels had higher antifungal effects than other HEMA-based cryogels. Within the scope of these results, the idea emerged that N-Vinylformamide addictive PHEMA-based cryogels can be conidered as a wound dressing material or antifungal drug loading systems against fungal infections in biotecnological fields. These studies could be updated with this idea following studies.

CONFLICT OF INTEREST

No conflict of interest was declared by the authors.

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