

Gelatin Cryogels Enriched with Tea Tree Oil (*Melaleuca alternifolia*): Characterization and Antibacterial Properties

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Research Article

Keywords: tea tree oil, *Melaleuca alternifolia*, cryogel, gelatin, antibacterial, natural biomaterial

Posted Date: January 30th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-2510166/v1>

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Abstract

Cryogel, which is a frequently used polymer material in recent years, has begun to be evaluated together with alternative treatment methods. Especially, therapeutic effectiveness of cryogels has been gradually increased with the contribution of biocompatible materials and plant ingredients. Natural polymers are known for their high biocompatibility, so there are many studies focusing on the relationship between structure and function in plant-based biopolymers. As a result, various dressing materials including gelatin, alginate, chitosan (CS), inorganic materials have been developed as biopolymers. In this study, gelatin-based cryogels containing three different concentrations of *Melaleuca alternifolia* (tea tree plant) oil (TTO-GCry) were synthesized under the specified conditions and characterization studies were carried out. Their antibacterial activity was evaluated by using two bacterial strains (*E. coli* and *S. aureus*). As a result of the evaluation, the antibacterial effectiveness of the developed cryogels on *E. coli* was between 21–50%, and on *S. aureus* it was between 28–72%. It can be stated that an increase in the amount of tea tree oil within the cryogel leads to an increase in its antibacterial effectiveness. This cryogel, which has antimicrobial properties, is composed of natural materials and has an environmentally-friendly structure. Its macroporous structure allows it to be used for multifunctional purposes, especially as a hemostatic agent in emergency situations and as a wound dressing material in wound healing.

1. Introduction

The origin of the word cryogel, which is a type of hydrogel, is based on Greek. In Greek, kri'os (kryos) means ice or freezing [1]. Unlike other known gels, cryogels are materials that polymerize in a cold environment [2]. Cryogels offer many advantages thanks to their interconnected large pores, hydrophilic structure, not requiring toxic substances and being mechanically durable as well as having a flexible structure. Cryogels, which have a history of approximately 50 years, are hydrophilic polymers with interconnected large pores synthesized at sub-zero temperatures (Fig. 1) [3]. These gels can be obtained by forming homogeneous or heterogeneous polymer networks that are physically or chemically cross-linked [4]. The highly interconnected and open macroporous structures of cryogels have been crucial for a variety of biomedical applications. In addition, cryogels can be synthesized in very different shapes and properties by using different precursors and applying different chemical reactions [5, 6, 7]. Cryogelation (or sub-zero temperature gelling) is a promising new technology that allows controlled formation of large-pore hydrogels [8].

Cryogels are heterophase systems in which the solvent is both in the structure of the polymer and in the macropores, unlike other generally known gels. Other known gels are homophase systems and often have micropores [9]. Cryogels, which are included in the class of hydrogels due to the use of water as a solvent during their synthesis, have more elasticity than traditional hydrogels. In addition, interconnected pores are less common in hydrogels than in cryogels [10]. Due to the smaller pore size of hydrogels compared to cryogels, the swelling rate of hydrogels is lower than of cryogels [11]. Hydrogels are mechanically less durable gels compared to cryogels, which causes cryogels to be preferred over hydrogels in many studies [12]. Furthermore, the larger pores of cryogels offer a remarkable feature in terms of processing

particulate liquids and cell suspensions [8]. In wound treatments that require controlled release, it also provides a suitable structure for substances such as oil and drugs that can increase healing.

The use of cryogels in biomedical applications has recently increased rapidly. The highly interconnected and macroporous structures of cryogels have been crucial for a variety of biomedical applications. Their large porosity, high water holding capacity, interpore connection and stability provide cryogels compatibility with biological materials [10]. Solvent crystals are used as porogens in the process of cryogel synthesis. When ice crystals melted, large interconnected pores are formed in the cryogel structure (Fig. 1). Cryogelation is a method that is generally considered more efficient in terms of time and resources compared to other techniques for producing macroporous gels. As Henderson et al. [13] have noted, it is easy to adjust various parameters to optimize cryogels for their intended use. Cryogels have several other benefits, one being their simplicity in production and often using water as a solvent, which makes the biomaterials more cost-effective and less harmful to the environment [10].

Gelatin, a hydrolyzed collagen, is one of the important materials known to have been used in ancient times of human history. This material, which can keep a large amount of solvent in its pores or in the interstitial spaces in its structure, can reach its maximum dimensions by swelling completely when exposed to a thermodynamically suitable solvent (water or any biological fluid) [14]. It has been scientifically proven that gelatin is biocompatible, flexible and stable, and can be modified. Gelatin consists of proline, glycine and hydroxyproline amino acids. As glycine in its structure allows cells to adhere, gelatin is widely used in biopolymer materials. In addition, tissues have the ability to metabolize gelatin, which does not cause any immune reaction [15]. This allows gelatin to contribute to the biocompatibility of polymeric materials. In a study in which gelatin-based cryogel synthesis was performed to develop tissue scaffolds, it was observed that gelatin increased the biocompatibility and cell renewal feature of the synthesized cryogel [16]. Due to all these properties, gelatin is included in the structure of tissue-compatible polymer materials in many studies.

The high biocompatibility of natural polymers has facilitated a greater focus on plant-based biopolymers and their structural and functional properties, leading to more research in this field. Biopolymers derived from plant polysaccharides such as cellulose, hemicellulose, lignin, starch, and pectin, and plant proteins like soy are being increasingly utilized in therapeutic and biotechnological research. High compatibility of these natural polymers with the extracellular matrix provides us with a field of use in new studies with the extracellular matrix. However, flavonoid or biflavonoid polyphenolic vegetable polymers found in plants can also be used in therapeutic studies with their antioxidant and antimicrobial properties [17].

Chemical compounds from plants can be divided into primary and secondary metabolites (phytopharmaceuticals) based on their direct involvement in development, growth, or reproduction. Many terrestrial plants, terpenoids (plant volatiles, carotenoids, steroids, etc.), phenolics (tannins and lignins, etc.) and nitrogen-containing compounds are important secondary metabolites. Botanical excipients are organic substances originating from different parts of a plant such as cell walls, stem exudates, and seaweeds [17]. It is possible to increase the effectiveness of the wound dressing with the therapeutic

effect of plants. In the literature, plant extracts/active substances have been incorporated in to wound dressing materials during the polymerization stage [11, 17]. At present, various dressing materials including collagen (Col), silk fibroin (SF), alginate, chitosan (CS), inorganic materials have been developed for many purposes [17, 18].

When we look at the literature, there are very few studies on the synthesis of plant-derived cryogels. In a study [19] herbal infusion oil (*Hypericum perforatum*, HP) containing chitosan (CS) cryogel was produced as a wound dressing material to be used in the wound healing process. The main aim is to obtain a potential polymeric material with the use of therapeutic oils to promote tissue formation and accelerate wound healing. As a result of the study, it was understood that the use of HP-loaded chitosan cryogel scaffolds as a surface dressing for the healing of long-term wounds is a promising option.

In another study by Yıldırım and Küçük [20], chitosan and gelatin-based surface wound dressing with St. John's Wort added (*Hypericum perforatum*) was prepared. According to the study, it was understood that *Hypericum perforatum*-based surface dressing has the potential to heal. In a study conducted in 2022, antibacterial gelatin-chitosan cryogels containing clove oil were designed [21]. In this study, a bioactive cryogel was developed using gelatin, chitosan and clove oil. According to the study, it was concluded that cryogel can be used as a biocompatible dermal substitute with antibacterial properties.

Melaleuca alternifolia (tea tree) is a plant that grows in Australia and New Zealand and exhibits strong anti-inflammatory and antimicrobial properties with its terpinen-4-ol and 1,8-cineole contents in its oil. Tea tree oil has been found to possess antioxidant properties, and it has been reported to have a wide-range of antimicrobial activity against various types of skin and mucosa infections such as bacteria, virus, fungus and protozoa [22]. Several studies have shown promising antibacterial effects of *Melaleuca alternifolia* or tea tree essential oil. A study by Edwards-Jones et al [23] evaluated the antibacterial activities of Patchouli, tea tree (*Melaleuca alternifolia*), geranium, lavender essential oils and Citricidal™ (grapefruit seed extract), alone and in combination, against three *Staphylococcus aureus* strains. Tea tree oil is renowned for its antiseptic properties, and in this study it was found to be particularly effective in inhibiting the growth of MRSA (Methicillin-resistant *Staphylococcus aureus*) when used alone.

A study by Chin and Cordell was conducted with 10 participants of different age groups and genders. In the in vivo study, it was observed that the wound dressing made with *M. alternifolia* shortened the healing time compared to the control group, in this case, the results of the previous in vitro study of *M. alternifolia* were also supported in vivo [24]. This situation leads to the conclusion that *M. alternifolia* plant can be used as a support in wound healing. In a study conducted in 2015, antidemogenic hydrogels containing tea tree oil nanocapsules and nanoemulsions were developed for wound healing [25]. In this study, it was aimed to evaluate the in vivo efficacy of hydrogels developed in protecting skin damage caused by UVB effect and cutaneous wound healing. Healing on the cutaneous wound type was evaluated in rats, and it was observed that the oil increased the biological activity in the healing process, and reduced the skin damage and edema caused by UVA-induced radiation.

In our study, we aimed to synthesize a plant-based cryogel using the therapeutic compounds found in *Melaleuca alternifolia* oil. Our goal was to create a plant-derived cryogel that could be used for rapid and effective first aid in emergency situations. Moreover, our aim was to contribute to the literature with a new acute wound healing and antibacterial polymer material. To the best of our knowledge, there is no previous study that has reported the use of tea tree oil in a cryogel dressing.

For this purpose, cryogels containing tea tree oil at different concentrations were synthesized and characterization studies were carried out on these cryogels. With the aim of understanding the properties, the gel fraction yield (%), swelling ratio (%), and macroporosity degree (%) were calculated for the cryogels. GCMS-Headspace (Gas Chromatography Mass Spectrometer) analysis was performed to detect the presence of tea tree oil in the cryogel. Then, FTIR (Fourier Transform Infrared Spectroscopy) analysis was performed and the macro-porous structure of the cryogel was visualized using SEM (Scanning Electron Microscope).

2. Materials And Methods

2.1. Materials

Tea tree oil, which is used in the synthesis of cryogels during polymer production, is 100% pure and obtained from a commercial company (Casel Farmacy Cahit Selimoğlu Pharmaceutical Industry and Trade Limited Company). HEMA (Sigma, St Louis, USA) was used as the monomer, while PEGDA (Sigma, St Louis, USA) used as the crosslinker. N,N,N',N'-tetramethylene diamine (TEMED) was used as the reaction accelerator. Ammonium persulfate (APS) (Merck, Darmstadt Germany) was used as the reaction initiator. Gelatin (from pigskin, Type A) and glutaraldehyde (25%) was obtained from Sigma (based in St. Louis, USA). Glutaraldehyde was used as a crosslinker for the gelatin.

Gel-PHEMA cryogel synthesis were performed at Hacettepe University Biology Laboratory. Antibacterial analyzes were carried out in the laboratories of Hacettepe University Department of Biology.

The alcohol used for sterilization and dehydration of SEM samples in the studies was obtained from Riedel de haen (Seelze, Germany). Hexamethyldisilazane (reagent grade, $\geq 99\%$, HMDS) used in preparing SEM samples was purchased from Sigma-Aldrich (St Louis, USA).

2.2. Synthesis of TTO GCry Cryogels

At the synthesis stage of the cryogel, 0.05 g gelatin with 13.5 distilled water was dissolved and different concentrations of tea tree oil (25, 50 and 100 μL) were added by drop method and mixed with a magnetic stirrer together with the control group (1 hour). Then, monomer HEMA (1.3 mL) and crosslinker PEGDA (0.506 mL) were added. Then, 0.02 g of the polymerization initiator APS was added followed by adding 25 μL of TEMED. Within 1 minute, it was poured into between two glasses which has been prepared before. Then, it was put in the freezer at -22°C and kept in the freezer for 24 hours. At the end of 24 hours,

it was kept at room temperature. After that, 8 mm diameter-sections were taken and characterization was started. The cryogels were kept in distilled water at 4°C until needed for use.

Since it was observed that the tea tree oil concentration added more than 100 µl impairs the stability of the cryogel, the maximum added amount was determined as 100 µl.

2.3. Characterization of cryogels

2.3.1. Swelling studies

The weights of swollen cryogels, which had reached equilibrium swelling by absorbing water, were measured ($m_{\text{swollen gel}}$). The water was then removed from these cryogels and their weight (squeezed gel) was measured. Then, after the gels were dried in an oven at 55°C (1 hour), the weight of the dry cryogels was measured as the dry gel weight ($m_{\text{dried gel}}$). The gelation efficiency was calculated with the following equations.

$$\text{Gel fraction yield (\%)} = (m_{\text{dried gel}} / m_t) \times 100 \quad (1)$$

The m_t value in this formula; refers to the total mass of monomers in the polymer mixture.

The following equation was used to determine the swelling ratio (%) of cryogels:

$$\text{Swelling ratio (\%)} = (m_{\text{swollen gel}} - m_{\text{dried gel}}) / m_{\text{swollen gel}} \times 100 \quad (2)$$

In order to calculate the total volume of supermacropores in cryogels, the equation given below is used to calculate the amount of macropores.

$$\text{Macroporosity degree (\%)} = (m_{\text{swollen gel}} - m_{\text{squeezed gel}}) / m_{\text{swollen gel}} \times 100 \quad (3)$$

2.3.2. Fourier Transform Infrared Spectroscopy (FTIR)

Spectra of dry samples (TTO 0, TTO 25, TTO 50, TTO 100), gelatin and pure tea tree oil samples were taken with FTIR (Jasco FT/IR-6600typeA, Forensic Chemistry Laboratories, Forensic Sciences Institute of the Turkish National Police Academy). The samples were examined in the wavelength range of 400–4000 cm^{-1} . It was compared with the results found in the literature [26].

2.3.3. Gas Chromatography Mass Spectrometer (GCMS-Headspace)

In order to understand whether the main metabolite of tea tree oil (terpinen-4-ol) is included in the structure of the synthesized cryogel in the study, GCMS-Headspace analysis was performed (Agilent 5977B GC/MSD and Agilent 7697A Headspace Sampler in Forensic Chemistry Laboratories, Forensic Sciences Institute of the Turkish National Police Academy). The study in the literature was used to determine the method to be used for the analysis [27]. TTO0, TTO25, TTO50 and TTO100 GCry-cryogel

samples cut in 1 mm dimensions were placed in each 20 ml autosampler vial without any thawing. Then it was placed in GCMS-HS. General adjustments were made with MSD- Mass Hunter program. Then comparisons were made with MSD Data Analysis and Quantitative Data Analysis Software.

2.3.4. SEM Analysis

SEM analyzes was performed at Hacettepe University Advanced Technology Research and Application Center (HUNITEK).

Cryogels were first fixed in glutaraldehyde solution. It was then dehydrated using alcohol and HMDS. In this process, 1 ml of 2.5% (v/v) glutaraldehyde solution was added onto each cryogel of the same size (each, 6 mm in diameter) and the cryogels were kept in the dark for 30 minutes. After fixation, SEM samples were washed with 1mL of PBS. For the dehydration process, the cryogels were sequentially in 30%, 50%, 70% and 100% (v/v) ethanol solutions for 5 minutes; and incubated in hexamethyldisilazane (HMDS) for 10 minutes. Afterwards, the cryogels were dried at room temperature and coated with gold-palladium to make SEM samples ready for imaging.

2.3.5. Antibacterial Analysis

For antibacterial studies, both liquid and solid medium were prepared. Liquid Luria Bertani (LB)-Brot medium and solid Agar growth medium were obtained. *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) strains were used in this study. These two strains were used to see the effect of TTO GCry cryogels on both gram positive and gram negative bacteria. A control group was formed for each of the 2 groups in bacterial cultivation. For *E.coli* and *S. aureus*, 1 ml of medium was added to 2 sterile eppendorfs. Sterile media and eppendorfs were inoculated. Bacteria were incubated with the medium for 18–24 h at 37°C at 150 rpm.

Then, for each bacterial group (*E.coli* and *S.aureus*), 100 µl of bacteria and 900 µl of medium were inoculated into eppendorf tubes (TTO0-Ec,TTO25-Ec,TTO50-Ec and TTO100-Ec, Control-Ec, TTO-Ec, TTO0-Sa, TTO25-Sa, TTO50-Sa, TTO100-Sa, Control-Sa, TTO-Sa). For the colony count experiment with agar medium, serial dilutions up to 10^{-9} were made (100 µl bac/900 medium) to *E.coli* and *S.aureus* bacterial groups incubated for 24 h at 37°C. 10^{-9} diluted eppendorfs were planted on agar medium by spreading method. It was kept at 37°C for 24 h. Colony count was performed 1 day later. The following formula was used for colony counting:

$$N(\text{Total number of microorganisms}) = \frac{(\text{Number of colonies} \times \text{Dilution Factor})}{\text{Volume (mL)}(\text{Amount plated microorganisms})} \quad (4)$$

Measurements were taken from 10^{-9} diluted eppendorfs and values were measured by the spectrophotometer at 620 nm. Then, the correlation relationship between the colony count method and UV-VIS spectrophotometer and measurement method was calculated for comparative analysis (Python

3.0, $p \leq 0.05$). According to the results, the antibacterial activity (%) of TTO GCry groups on *E.coli* and *S. aureus* strains was calculated.

3. Results

3.1 Synthesis of TTO GCry Cryogels

In the study, TTO GCry cryogels containing different concentrations of TTO, 0, 25, 50, 100 μl , were kept at -22°C for 24 hours and then kept at room temperature to melt.

It has been observed by repeated experiments that all cryogels are structurally successful. Then, 8mm diameter sections were taken from the cryogels (Fig. 2). Swelling weights, squeezed weights and dry weights of cryogels were measured in 3 replicates for swelling data. The arithmetic average of the measurements taken with 3 replicates was used. The total polymer percentages and forms of the obtained cryogels are shown in Table 1.

Table 1
The total polymer percentage (%w/v) of the obtained cryogel groups, the amount of tea tree oil added and the resulting cryogel form

Sample Name	Total Polimer (%,mg/ml)	Oil Content μl	Form
TTO0 GCry	14	0	Off-white, flexible
TTO25 GCry	16	25	Off-white, flexible
TTO50 GCry	16	50	Off-white, flexible
TTO100 GCry	15	100	Off-white, flexible, more stabile

3.2 Swelling Results

In the study, gel fraction yield (%), swelling ratio (%) and macroporosity degree (%) of the samples are shown in the graphics. (Table 2) According to the gel fraction yield results, TTO0 GCry showed the highest yield with 80%. TTO25 GCry 70%, TTO50 GCry 73%, TTO100 GCry 70% gelling efficiency. It can be said that the inclusion of tea tree oil in the structure slightly reduces the yield, but it is not directly proportional to the increase in the amount. Considering the swelling rates, the values are generally close to each other, between 88,5% and 91%. In fact, TTO100 GCry (100 μl tea tree oil) with the highest amount of tea tree oil had the highest swelling rate. When looking at the amount of macroporosity degree, the results are found as 72% for TTO0 GCry, 74%, for TTO25 GCry. 75% for TTO50 GCry and 71% for TTO100 GCry. The highest macroporosity was seen in TTO50 GCry.

Considering the swelling properties in general, it is seen that the addition of tea tree oil does not negatively affect the swelling properties of the cryogel and does not have any negative effect on the stabilization of the cryogel, although it is incorporated into the structure.

Table 2
Swelling Results of cryogels

Type of cryogel	Gel fraction yield (%)	Swelling ratio (%)	Macroporosity degree (%)
TT00 GCry	80	88,5	72
TT025 GCry	70	89	74
TT050 GCry	73	88	75
TT0100 GCry	70	91	71

3.3 Fourier Transform Infrared Spectroscopy (FTIR)

In this study, we evaluated the FTIR results for each cryogel separately and together. Apart from this, we also included spectrum comparisons of gelatin and pure tea tree oil (Fig. 3–4).

Previous studies and literature have established that the bands around 2920 cm⁻¹ are a result of both asymmetric and symmetric C-H stretching vibrations, and the broad peak at around 3300–3400 cm⁻¹ is indicative of O-H stretching vibrations (Fig. 4). The presence of high intensity band at 1720 cm⁻¹ indicates the presence of C=O stretching vibrations. Amides are characterized by absorption bands resulting from N-H and C-O stretching vibrations [28].

When we examine the infrared spectrum of the GCry cryogel, gelatin-specific amide I band is seen at 1641-44 cm⁻¹ (C = O stretch), amide II bands at 1542-43 cm⁻¹ (double bending with C-N stretching) and (CH₂ bending frequency) at 1448-51 cm⁻¹. These bands were found to be compatible with the studies in the literature. The amide III bands originating from gelatin and PHEMA are seen at 1243–1244 cm⁻¹ and 1071-74 cm⁻¹ (N-H bending and C-O stretching) (Fig. 3–4)[29]. The detection of amide bands indicates that the gelatin was incorporated into the structure of the GCry cryogel (Fig. 3, Fig. 4-c). Looking at Fig. 4(a) and (b), it is seen that as the amount of TTO in cryogels increases, the length of lines at certain wavelengths also increases. Characteristic C-H bond of terpenoids in aromatic oils is seen around ~ 2900 cm⁻¹, C = O bond (~ 1700 cm⁻¹), a large O-H stretch is around ~ 3400 cm⁻¹ and C-O stretching is around ~ 1100 cm⁻¹ [30]. Extensions in these wavelengths are seen in the spectrum of tea tree oil itself. As a result, the elongation at the same wavelengths increased as the tea was added, indicating that the oil is in the cryogel structure and affects the absorbance. The peak of around ~ 1647 cm⁻¹, a specific point for tea tree oil, appears to become more pronounced as the amount of tea tree oil loaded into the cryogel increases in Fig. 4(a) [31].

3.4 Gas Chromatography Mass Spectrometer (GCMS-Headspace) Results

The tea tree oil metabolites in the structure of TTO GCry cryogel are shown in Fig. 5 in the GCMS-Headspace Mass Spectrum. In the first stage of the study, the results for the cryogels loaded with the

least amount of TTO (TTO25 GCry, 25 µl loaded cryogel) showed that the tea tree oil was entrapped in the cryogel structure. When we look at the past studies and compared with the ion chromatogram of terpinene 4-ol, the main metabolite of tea tree oil, it was seen that these ions are present in the structure of GCry cryogel [32–33]. For example, the Q (m/z) value (qualification ion) of terpinene 4-ol, the main metabolite of tea tree oil, is 71, q1 (m/z) and q2 (m/z) values (qualifier ions) are 93 and 111, respectively (Fig. 5(b)). The ion values of other metabolites are shown in the spectrum of cryogel [34]. Accordingly, it can be said that tea tree oil and its metabolites, including its main metabolite terpinene 4-ol, participate in the structure of TTO GCry cryogel.

3.5 SEM Results

In this study, surface analyzes were carried out to observe the structural properties of the cryogel [35] and to investigate the effects of the gelatin and tea tree oil used in the cryogel. When the SEM images of the PHEMA cryogels were evaluated, it was seen that macropores were formed in each cryogel as expected and the cryogels were successfully synthesized. According to SEM images, it was observed that the cryogel macropore sizes were more uniform with the addition of TTO. This uniformity allows the tea tree oil to spread evenly in applications such as its use for wound healing on the skin. (Fig. 6. a-f) This is also compatible with the previous oil-added cryogel work [19]. According to SEM analysis, average pore sizes range from 30–50µm for TTO0 GCry, 30–45µm for TTO100 GCry, and 15–50µm for Control-PHEMA (NO gelatin or TTO). Although the average pore sizes were the same, the addition of gelatin created a uniform effect, and the values closer to the average were observed with the use of tea tree oil and gelatin together.

3.6 Antibacterial Analysis Results

For UV-VIS Spectrophotometer measurement, 100 µl of bacteria (1/10 dilution) was added from the bacterial stock to 900 µl of medium. For each bacterial species and group, a separate medium was cultivated and placed in eppendorfs. For this study, 20 eppendorfs were used, including the control group and tea tree oil (5 µl tee tree oil for 900 µl medium) for each bacteria. Each group was diluted to 10^{-9} . All groups were incubated for 18–24 h at 37°C at 150 rpm. Colony count and spectrophotometric analysis were performed after 24 hours.

After the colony count in all groups was completed, according to the correlation matrix between the absorbance values at 620 nm and the calculated colony numbers, it was observed that there was a high linear positive correlation between the two measurement values, ranging from 0.99-1. (Python 3.0, $p \leq 0.05$) (Table 4) According to these data, the antibacterial effect (%) of all TTO GCry groups on *E.coli* and *S. aureus* is shown in Table 5. The lowest antibacterial activity was seen in cryogels without the addition of tea tree oil (GCry cryogels- TTO0-Ec and, TTO0-Sa), while the highest antibacterial activity was observed in cryogels with 100 µl of tea tree oil added. (GCry cryogels- TTO100-Ec, and TTO100-Sa). TTO100 GCry was found to have 50% antibacterial activity on *E.coli* and 72% on *S.aureus*. It was observed that the antibacterial activity increased as the amount of tea tree oil increased. (Table 5).

In a previous study, TTO MIC values calculated by the Broth microdilution method were found to be 0.25% (v/v) for *E. coli* and 0.50% (v/v) for *S. aureus* [36]. In our study, it was observed that 0.50% (v/v) value in agar plate cultivation, which we used as a positive control, had 100% antibacterial effect for both bacteria. Since the gel structure was deteriorated when we added TTO exceeding 100 µl to the cryogel we synthesized, the maximum antibacterial activity was evaluated with a TTO value of 0.13% (v/v) (*E.coli* 50%, *S. aureus* 72%).

Table 4 Correlation relationship between colony count (10¹⁰ cfu/ml) and 620 nm absorbance values of TTO GCry groups (*E.coli*-EC, *S.aureus*-SA)

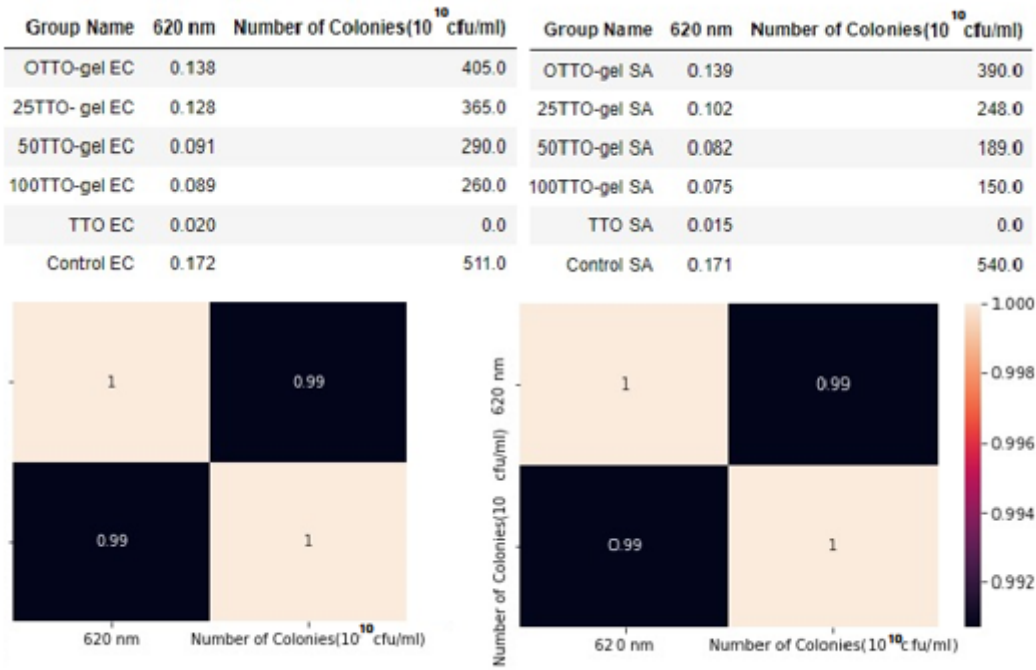


Table 5
Antibacterial activity of TTO GCry groups groups on *E.coli* and *S. aureus* strains (%)

Antibacterial activity %	TTO0 GCry	TTO25 GCry	TTO50 GCry	TTO100 GCry	TTO (0,5%, v/v)
E.coli	21	29	43	50	100
S. aureus	28	54	65	72	100

4. Conclusion

In recent years, research on polymer materials has increased considerably, and with biotechnological developments, it has begun to find a place in the fields such as wound healing and tissue engineering. Hydrogels, one of these materials, also have a wide range of uses. Among the many types of hydrogels, there are various versions in the literature of cryogels that are exposed to low temperatures during the

polymerization stage, thereby obtaining higher pore sizes. It is aimed to obtain more efficient and effective results by combining plant-based alternative treatment methods and biotechnological developments.

In this study, a PHEMA cryogel synthesis was performed with gelatin and *Melaleuca alternifolia* (tea tree oil) because of its antibacterial and biocompatibility properties. As a result of that, its characterization and antibacterial properties were evaluated. Accordingly, it was observed that TTO GCry cryogels were successfully synthesized by the addition of tea tree oil. According to GCMS-Headspace Mass Spectrum analysis, tea tree oil metabolites, including its main metabolite terpinene 4-ol, were found to participate in the structure of TTO GCry cryogel. These results were also supported by FTIR analysis.

When the swelling properties were evaluated, the addition of tea tree oil increased the swelling rates, although the gel fraction yield decreased from 80–70%. This can be interpreted as the tea tree oil added during the synthesis phase may increase the pore size. Also it therefore slightly increases the swelling rate. TTO50 GCry had the highest amount of macropores (75%), while TTO100 GCry had the highest degree of swelling (91%). In antibacterial evaluation, using two different measurement methods (serial dilution and spectrophotometric analysis) can increase the possibility of more accurate evaluation of the results. As a result of antibacterial analysis, tea tree oil of TTO100 GCry was found to have maximum antibacterial effect on *S. aureus* and *E. coli*. However, it has been observed that it has a higher antibacterial effect on *S. aureus* growth.

In future studies, more tea tree oil loading can be applied to the cryogel developed with a different formula. Thus, the antibacterial activity can be increased. Although it is mentioned in the literature that the toxic effect of tea tree oil is very low unless taken orally, biocompatibility evaluation can be made [37, 38, 39].

In this case, it can be said that this cryogel developed with tea tree oil and gelatin content can offer an alternative treatment approach in many applications.

Declarations

Funding

This study was not funded by any institution. The studies were carried out in Biochemistry Laboratory, Department of Biology Hacettepe University.

Acknowledgements

We would like to thank the Institute of Forensic Sciences, Chemistry Laboratory, Turkish National Police Academy, for the use of the device in the analyzes performed with FTIR.

Author Contribution

SV performed cryogel synthesis and characterization, antibacterial studies, and contributed to finalizing the manuscript. GB supported antibacterial studies and cryogel characterization. IPD contributed to the planning of the study, the control of the experimental process, the revision and finalization of the manuscript.

Data Availability Statement

The datasets used and/or analyzed during the current study are used in another study. It can be forwarded upon request.

Conflict of Interest

The authors declare that they have no conflict interest.

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Figures

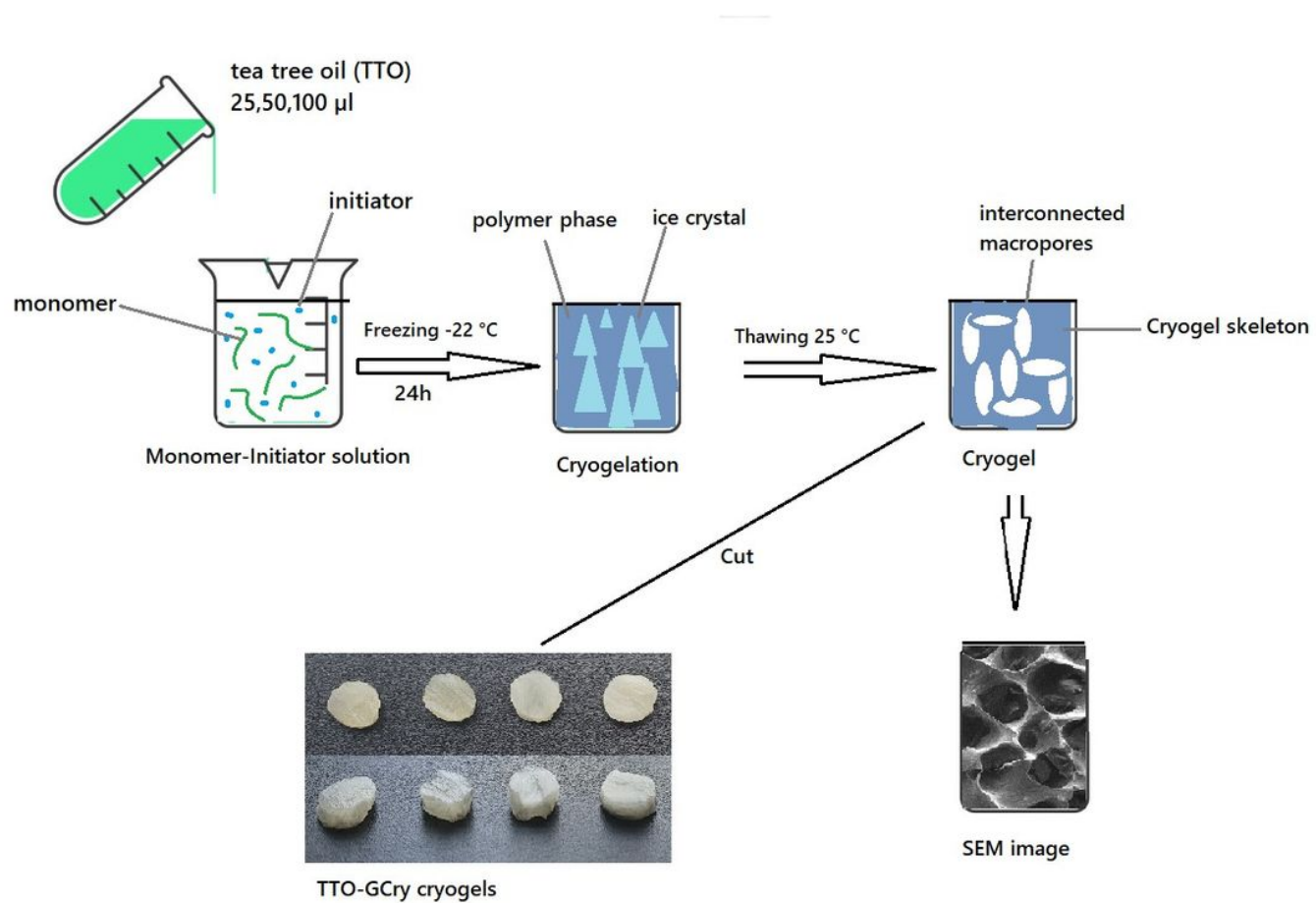


Figure 1

Schematic illustration of preparation and characterization of TTO-GCry cryogels.



Figure 2

TTO GCry cryogels containing different concentrations of TTO (0,25,50,100 μ l),

a) swollen cryogels and different concentrations b) dry cryogels and different concentrations

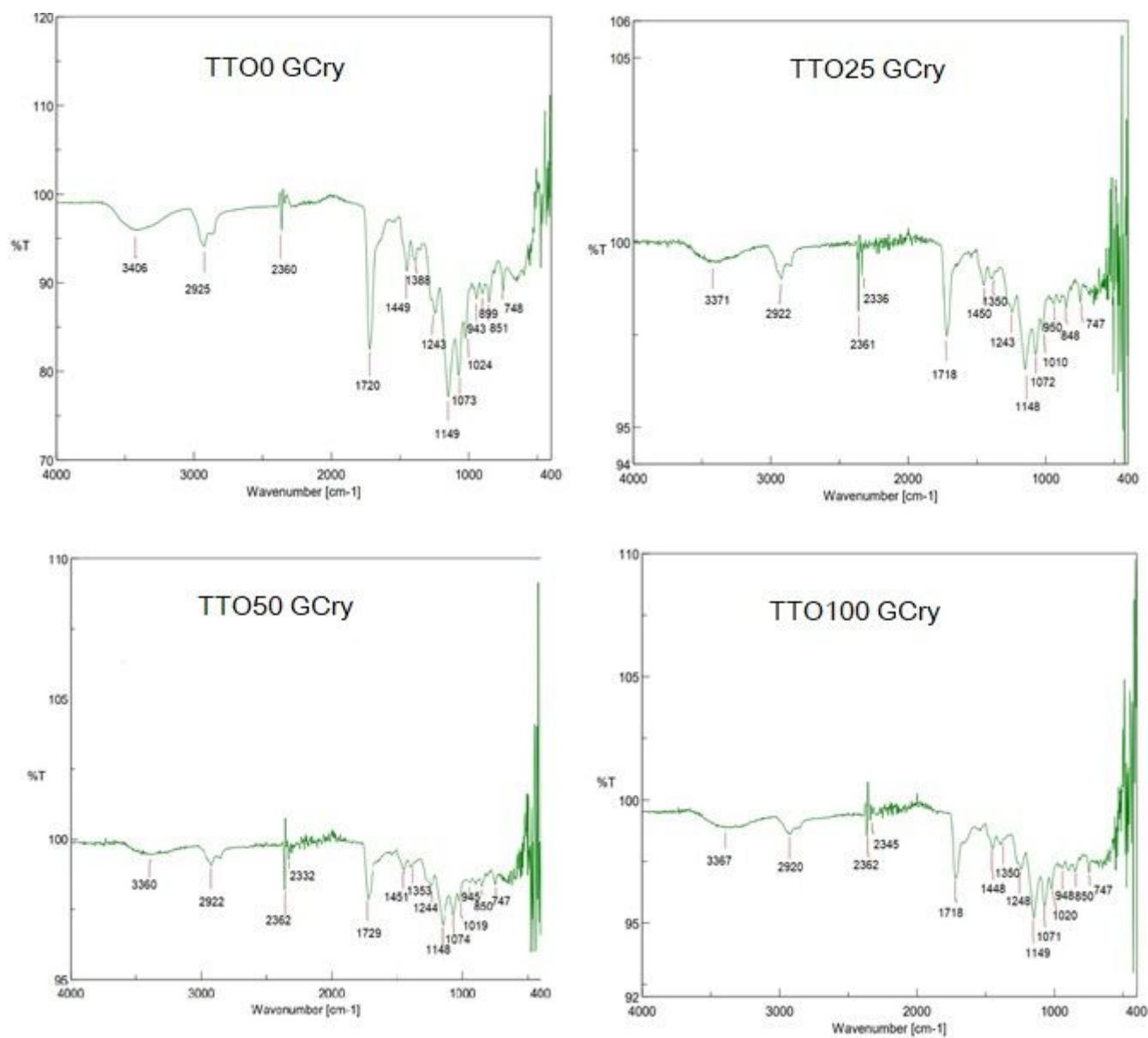


Figure 3

Spectra of Cryogels, $400\text{-}4000\text{ cm}^{-1}$, FT/IR-6600typeA (TTO0, TTO25, TTO50, TTO100 GCry cryogels, respectively)

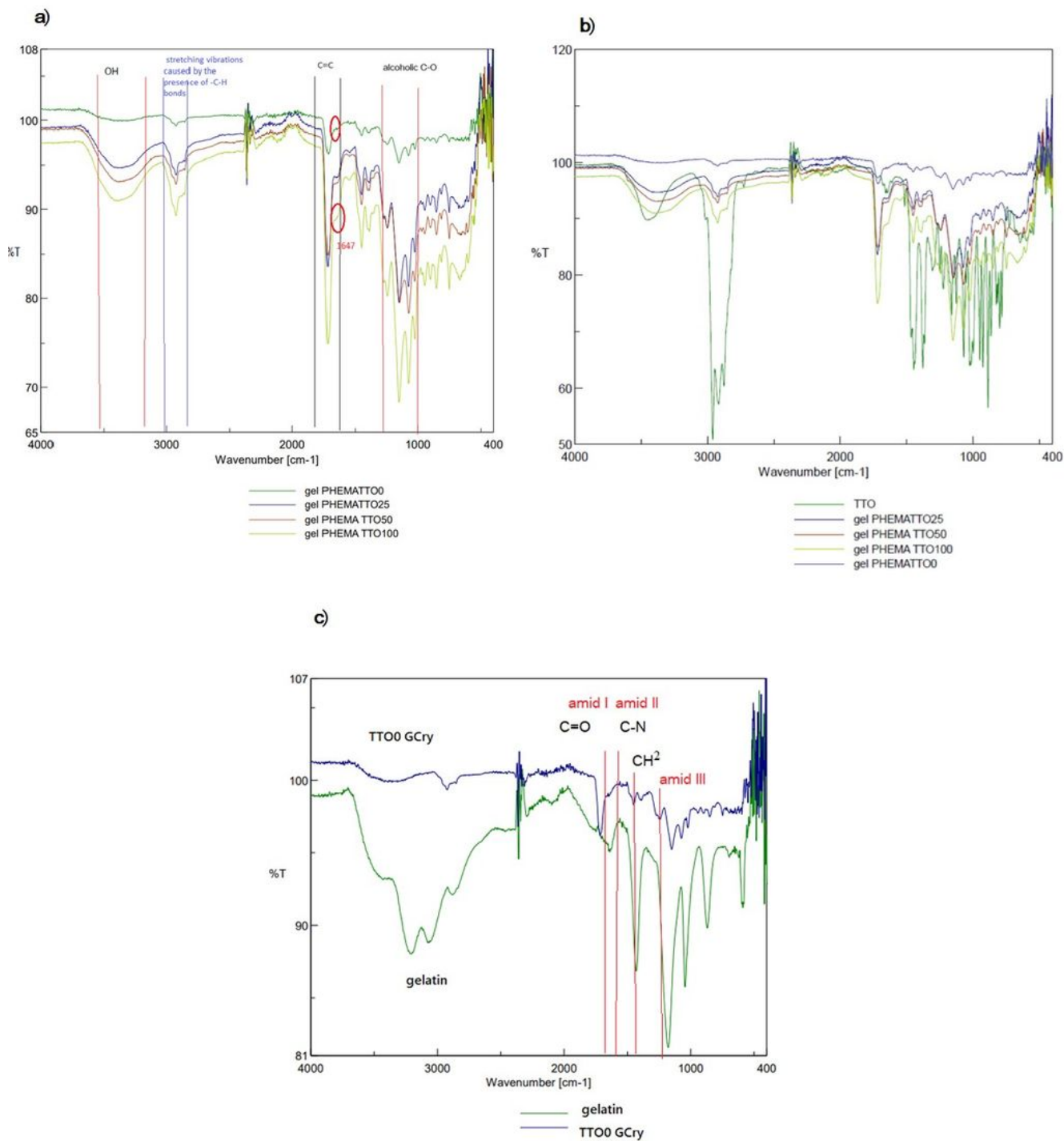


Figure 4

Comparative spectra of TTO GCry cryogels (400-4000 cm⁻², FT/IR-6600typeA), a) comparison of all cryogels, b) comparison of tea tree oil (TTO) and all cryogels, c) comparison of TTO0 GCry and gelatin

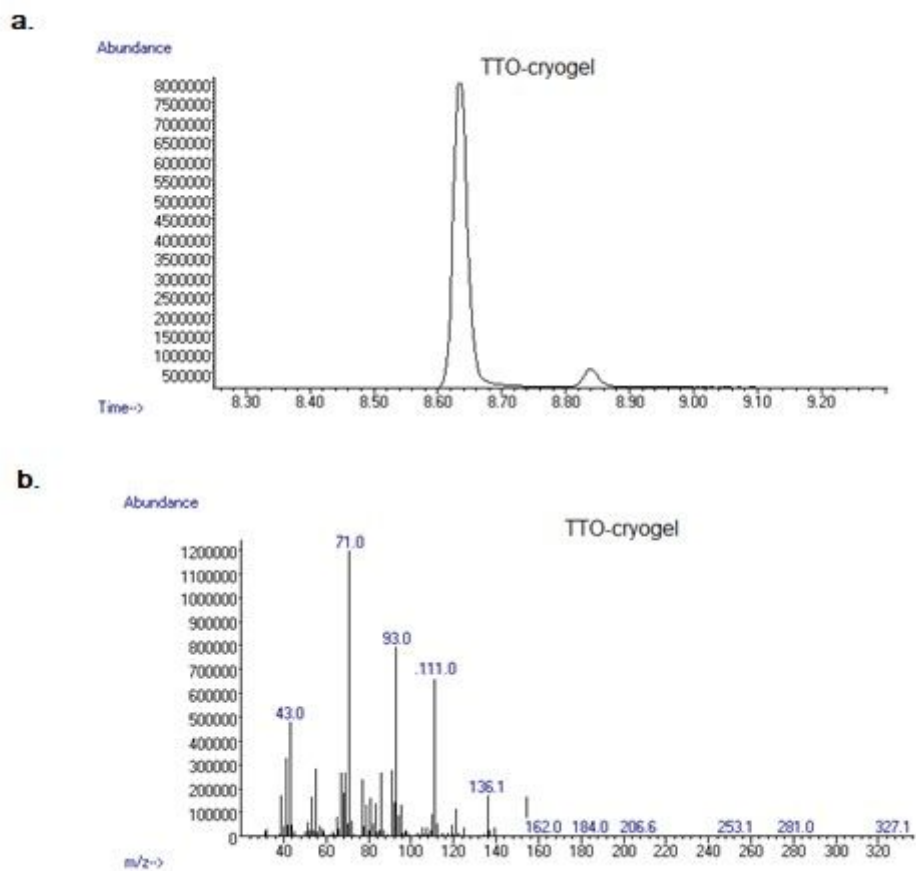


Figure 5

TTO- GCMS-Headspace Mass Spectrum of cryogel (peak value of terpinene 4-ol, for TT025 GCry), a. cryogel abundance/time spectrum (peak value of terpinen-4-ol) b. abundance-m/z spectrum, ion chromatogram of terpinen 4-ol)

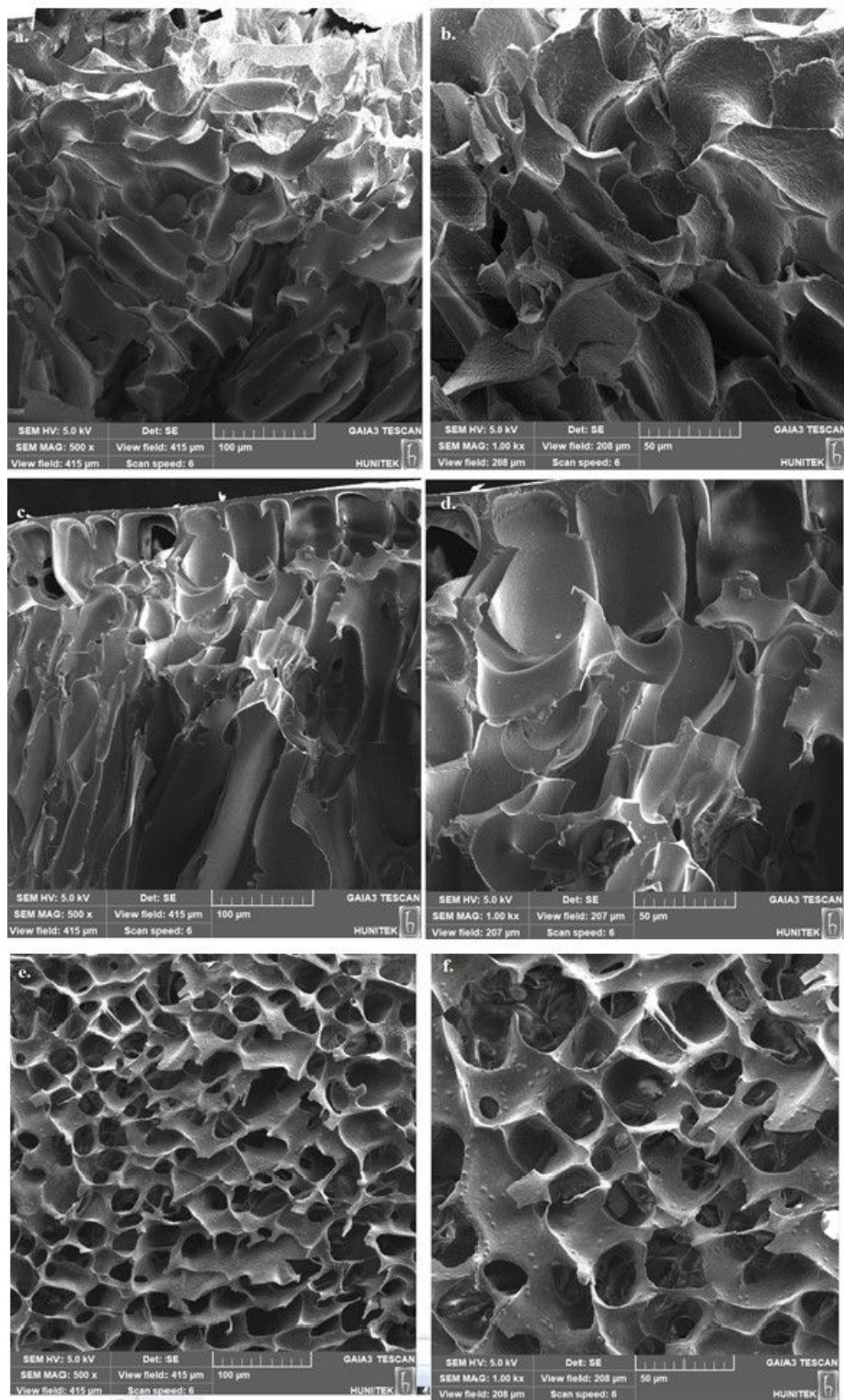


Figure 6

SEM images of PHEMA and GCry cryogels, a-b) TTO0 GCry, a. 500 X, bar 100 μm , b. 1000 X, bar 50 μm , c-d) TTO100 GCry, c. 500 X, bar 100 μm , d. 1000 X, bar 50 μm , e-f) Control-PHEMA cryogel(no gelatin or TTO added), e. 500 X, bar 100 μm , f. 1000 X, bar 50 μm