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Antimicrobial chitosan-sodium tetrafluoroborate ($NaBF_4$) hydrogels for topical applications

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ABSTRACT

Infection of a wound is one of the most important reasons delaying the recovery of an injured tissue. In this study, chitosan-based hydrogels were loaded with different concentrations of sodium tetrafluoroborate ($NaBF_4$) to fabricate an antimicrobial wound care system. Antimicrobial activity, and cytotoxicity of $NaBF_4$, and surface morphology, chemical bond structures and antimicrobial activity of Chitosan:$NaBF_4$ hydrogels against a broad spectrum of microorganisms including an antibiotic resistant specie were investigated. $NaBF_4$ showed higher antibacterial activity for gram-positive bacteria than gram-negative bacteria. MIC values of $NaBF_4$ were 3.906, 1.953, and 7.813 µg/µL for every gram-negative, gram-positive, and fungal species, respectively. Direct cytotoxicity of $NaBF_4$ on the L929 cell line was investigated by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. IC_{50} value after 24 h incubation was calculated as 3.2 µg/µL which is within the range of concentration with antimicrobial activity. The antimicrobial activities of chitosan hydrogels were investigated by disc diffusion method. Antimicrobial activity of hydrogel increased with increasing $NaBF_4$ concentration while high molecular weight chitosan-based hydrogel did not show antimicrobial activity. According to the results, group 1:3 (546.5mM $NaBF_4$ containing hydrogel) was enough to achieve broad spectrum antimicrobial activity and hydrogels prepared with this formulation can be used as a potential antimicrobial wound care product.

1. Introduction

Microbial contamination is a great concern, especially in terms of wound healing and biomedical implant fouling [1,2]. Presence of harmful microorganisms in the injured areas can cause various infections and these infections can prolong the healing process and may lead to tissue morbidity or sepsis depending on the severity of the infection. Moreover, the rapid development of antibiotic-resistant microorganisms complicates the situation [3,4]. For this reason, many researchers are interested in designing wound care systems that do not allow microbial contamination while accelerating the wound healing process. Designing a wound care system begins with the selection of the material. There are various polymeric materials used as wound dressing such as cellulose, gelatin, alginate, chitosan etc. [5-8]. Among these biopolymers, chitosan is one of the most widely used one due to its favorable properties such as hemostasis, biocompatibility, biodegradability, bacteriostasis and controlled drug release ability [9-11].

Chitosan is a linear natural polysaccharide obtained by partial deacetylation (>40%) of chitin, the major constituent of the exoskeleton of crustaceans, and comprised of glucosamine and N-acetyl-glucosamine monomers linked through β(1-4) bonds. Chitosan, which has many uses in its pure form, offers easy functionalization or modification by chemical and enzymatic processing due to the existence of hydroxyl (-OH) and amine ($-NH_2$) functional groups in its structure, and can be made suitable for use in many different areas by improving its physical and biological properties [12,13]. Chitosan, the second most abundant natural polysaccharide after cellulose, finds a wide range of applications in different medical and pharmaceutical devices due to its non-toxic, biocompatible, biodegradable and antimicrobial and antifungal properties [14,15].

Aside of its numerous advantages, chitosan is sensitive to water and has relatively low hardness and low durability that limits its use. In this context, crosslinkers are used to protect the biological properties of chitosan-containing biomaterials, to increase their mechanical strength and chemical stability, and to control their water permeability, solubility and swelling properties

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[16]. Crosslinking can be achieved either by formation of chemical bonds or physical crosslinking. So far, chemicals such as glutaraldehyde, tripolyphosphate, ethylene glycol, diglycidyl ether, diisocyanate and genipin have been used as chemical crosslinkers for chitosan. However, most of the synthetic cross-linkers are cytotoxic and may reduce the biocompatibility of the developed biomaterial. Among the chemicals, genipin stands out with its biocompatibility and high stability. Chitosan and genipin-containing biomaterials are used in many important areas such as preparation of elastic cartilage tissue material, production of carrier materials for controlled drug release, encapsulation of biological products and living cells, bio fabrication of muscle and vascular wall tissues, and production of wound dressing materials for humans and animals [17].

Chitosan is a water insoluble polymer. In order to induce the solubility of chitosan in water, an acidic media is required. The amino groups of chitosan are protonated by the acidic environment and the protonation of the amino groups present in the chitosan adversely affects the bonding forces between the polysaccharide chains. By increasing chitosan concentration or by adjusting the solution pH, gels can be formed by precipitation of chitosan chains. Glycerol phosphate is a basic salt that can be used as physical crosslinker. When a basic salt is added to a chitosan solution, pH of the solution increases by neutralizing phosphate groups, therefore solution remains liquid in these conditions. When temperature is increased to 37°C, due to hydrogen bonding, hydrophobic, and electrostatic interactions, original solution begins to be transformed into the gel matrix. This solution remains liquid below room temperature at pH values between 6.8 to 7.2 and turns into a gel when heated to body temperature which can be defined as heat-induced gelation which is very important for injectable gel applications [18-22].

In this study, glycerol phosphate and genipin have been used to obtain co-crosslinked chitosan hydrogels. These two physical and chemical crosslinkers have been used in a previous study and some advantages have been listed as reduced gelation time, improved mechanical properties and storage modulus due to physical and covalent crosslinking. Even co-crosslinked hydrogels display small pore size, and swelling properties were similar to those physically crosslinked ones [22].

Although chitosan is known as an antimicrobial biopolymer the antimicrobial efficiency changes with various factors such as type of microorganism, molecular weight, concentration, pH, temperature, ionic strength, reactive time etc. [23]. Therefore, researchers have been working on enhancing the antimicrobial property of chitosan by adding different antimicrobial agents [24-27]. Sodium tetrafluoroborate ($NaBF_4$), is a water-soluble molecule and releases high electronegative anion (BF_4^-) when dissociated into ions. Microbial cell walls have ionic charges and negatively signed ions may interact with microbial cell walls and alter membrane integrity which could be resulted by antimicrobial activity. To the best of the author's knowledge, there is no scientific study in the literature about broad spectrum antimicrobial activity and cytotoxicity properties of $NaBF_4$. In this study, the antimicrobial properties of high molecular weight chitosan hydrogels were crosslinked by using genipin and glycerol phosphate, and different concentrations of $NaBF_4$ addition were investigated for a potential wound care product.

2. Materials and Methods

2.1. Materials

High molecular weight chitosan was purchased from Sigma-Aldrich (USA). The degree of deacetylation (DD) and the molecular weight of the chitosan were >75% and between 310-375 kDa, respectively. Isomeric mixture of glycerol phosphate disodium salt hydrate and acetic acid glacial, puriss. 99.8-100.5% were purchased from Sigma-Aldrich (USA). Genipin was purchased from Challenge Bioproducts Co., Ltd. (Taiwan). $NaBF_4$ was purchased from Acros Organics (Belgium). Nutrient agar (NA), nutrient broth (NB), potato dextrose agar (PDA), and potato dextrose broth (PDB) were purchased from Sigma Aldrich. Ofloxacin, nystatin, and blank discs were purchased from Oxoid (UK). Cell culture chemicals were purchased from Biowest (France) unless it was stated. Microbial species used in antimicrobial activity tests, given in Table 1, were obtained from American Type Culture Collection (ATCC, USA). *Penicillium* sp. was kindly provided by the culture collection of Yeditepe University, Department of Genetics and Bioengineering. L929 mouse fibroblast cell line was also obtained from ATCC.

Table 1. Microbial species used for $NaBF_4$ and $NaBF_4$ incorporated chitosan hydrogels antimicrobial activity tests.

	Species	ATCC Code
Bacteria	*Eschericia coli*	25922
	Pseudomonas aeruginosa	27853
	Acinetobacter baumannii	9606
	Salmonella typhi	19430
	Klebsiella pneumoniae	13883
	Staphylococcus aureus	29213
	Methicillin-resistant *Staphylococcus aureus* (MRSA)	33592
Fungi	*Candida albicans*	10231
	Aspergillus niger	16404
	Penicillium sp.	-

2.2. Disk Diffusion Tests of $NaBF_4$

The antimicrobial property of $NaBF_4$ was determined by using the disk diffusion method described previously

[28]. $NaBF_4$ solution was prepared in 500 mg/mL concentration by using ultrapure water. On the other hand, 100 µL of 0.5 McFarland bacterial suspension were inoculated on the agar plate, for yeast and fungus 1 McFarland suspensions was used. NA and PDA were used for bacterial and fungal isolates, respectively. Blank discs were impregnated with 20 µL of $NaBF_4$ solution and placed on the inoculated plates. Ofloxacin and nystatin discs were used as positive control (PC) for bacteria and fungus, respectively. The plates were kept in incubators for 24 h at 36±1 °C for bacterial strains, 48 h at 36±1 °C for yeast and 72 h for fungus isolates at 27±1 °C to promote microbial growth. Inhibition zone diameters around $NaBF_4$ doped discs were measured to determine the antimicrobial activity.

2.3. Minimum Inhibitory Concentration of $NaBF_4$

The MIC values were determined according to the broth microdilution method described previously in a previous study [29]. Microbial species given in Table 1 were inoculated on NA for bacterial strains and for PDA of yeast and fungal species. Inoculated plates were incubated 24 h at 36±1 °C for bacterial strains, 48 h at 36±1 °C for yeast and 72 h for fungus isolates at 27±1 °C. Fresh grown cultures were used to prepare 0.5 McFarland microbial suspensions. Bacterial and yeast suspensions were prepared in sterile phosphate-buffered saline (PBS) and fungal suspensions were prepared in 0.5% (v/v) Tween 80 + PBS solution. On the other hand, $NaBF_4$ was dissolved in sterile ultrapure water with a concentration of 500 µg/µL in sterile test tubes. The 96-well plates were used for MIC tests, 200 µL from the stock solutions of $NaBF_4$ prepared at the concentration of 500 µg/µL was added into the first well. One hundred µL sterile ultrapure water was added to the sequential nine wells. Then, 100 µL from their serial dilutions was transferred into nine sequential wells. Then, 95 µL broth and 5 µL of each inoculum were added into each well. NB were used for bacterial species and PDB were used for fungal isolates. Two hundred µL pure broth was added into the 11^{th} wells as positive control and 12^{th} wells containing 195 µL broth and 5 µL of the inoculum was used as a negative control. Plates were incubated in a shaker incubator at 180 rpm at appropriate time and temperature. The lowest concentration of $NaBF_4$ inhibits the growth of microorganisms (MIC values), was determined using a microplate reader with absorbance (Abs) at 600 nm for bacteria and 530 nm for fungal isolates.

2.4. Minimum Bactericidal (MBC) and Fungicidal (MFC) Concentration of $NaBF_4$

MBC/MFC values were determined in accordance with the lowest concentrations of $NaBF_4$ which showed no microbial growth. Starting from the MIC values to higher concentrations 100 µL suspensions were spread on NA for bacteria, and PDA for yeast and fungi. Inoculated plates were incubated for appropriate time and temperature and the lowest concentrations of $NaBF_4$ that showed no microbial growth were recorded as MBC/MFC value [29].

2.5. Cytotoxicity of $NaBF_4$ by MTT Analysis

Cytotoxicity of $NaBF_4$ was directly determined in cell culture medium. L929, mouse fibroblast cell line, was used in cell culture studies. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) High Glucose containing 10% (v/v) fetal bovine serum (FBS), 1% (v/v) L-glutamine and 10 units/mL penicillin, 10 µg/mL streptomycin solution. L929 cells were seeded as 10^4 cells/well on 96 well plate and cultured at 37°C in 5% CO_2 atmosphere. The cells were cultured for one day to cover the surface. The day after, the stock solution of $NaBF_4$ was prepared in complete cell culture medium and diluted in cell culture medium between 100-0.098 mM. $NaBF_4$ containing cell culture media was applied to L929 cells (n=6). Cellular viabilities after 24 h $NaBF_4$ exposure were analyzed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) analysis. Used cell culture medium was aspirated and MTT containing culture media without FBS was added onto the cells. The cells were cultured in MTT containing media for 4 h at 37°C in 5% CO_2 atmosphere. At the end of the incubation period, used media was removed and formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Absorbance values were recorded at 570 nm reference to 690 nm with BMG Labtech, Spectrostar Nano, Germany. Cell viability is given as % viability of control cells cultured without $NaBF_4$.

2.6. Hydrogel Synthesis

High molecular weight chitosan was dissolved in 100 mL 1% (v/v) aqueous acetic acid solution in ultrapure water as 1 g/100 mL. This solution was left to mix for 24 h until it was clear. Unsolved solid particles were removed by centrifugation at 8000 rpm for 10 min. In order to prepare different concentrations of Chitosan:$NaBF_4$ (Ch:$NaBF_4$) hydrogels 10 mL of chitosan solution were separated into beakers and specific amounts of $NaBF_4$ was mixed with chitosan solutions as given in Table 2.

Table 2. Hydrogel synthesis conditions.

Ch:$NaBF_4$ (w/w)	Chitosan (g)	$NaBF_4$ (g)	$NaBF_4$ (mM)	GP (M)	Genipin (mM)
1:0*	0.1	0	0	0.12	1
1:1	0.1	0.1	182.2	0.12	1
1:2	0.1	0.2	364.3	0.12	1
1:3	0.1	0.3	546.5	0.12	1
1:4	0.1	0.4	728.7	0.12	1
1:5	0. 1	0.5	910.8	0.12	1

*Control hydrogel group without $NaBF_4$.

Then, glycerol phosphate (GP) and genipin were added into these solutions as crosslinkers, respectively. Polymer-crosslinker solution kept at 37°C for one day to complete the crosslinking process.

2.7. Characterization of Ch:$NaBF_4$ Hydrogels

Surface morphology of freeze-dried Ch:$NaBF_4$ hydrogels were characterized by Scanning Electron Microscopy (SEM, FEI, Quanta 650, USA) analysis. Samples were coated with Au-Pd for 60 seconds under argon atmosphere using plasma sputter before SEM imaging. The presence of specific chemical groups in the hydrogels were analyzed by Fourier Transform Infrared Spectroscopy (FTIR, PerkinElmer, USA) in the 4000-400 cm^{-1} wavenumber range.

2.8. Agar Disc Diffusion Method

Inhibition of microbial growth by Ch:$NaBF_4$ hydrogels were tested against microorganisms given at Table 1 by using agar disc diffusion method as described previously [30]. Briefly, 0.5 McFarland bacteria, yeast and fungi were prepared from fresh growth cultures. 100 µL of each microbial suspension were inoculated on NA for bacteria, and PDA for yeast and fungi. Ch:$NaBF_4$ hydrogels were cut out with a cork borer (approximately 7 mm diameter hydrogels were obtained) and placed on the inoculated agar. Inoculated plates were incubated for appropriate time and temperature for all microbial species and antimicrobial activity was determined by measuring the inhibition zone diameters.

3. Results

3.1. Antimicrobial Activity of $NaBF_4$

Antimicrobial activity tests were conducted against seven bacteria, one yeast, and two fungi species based on agar disk diffusion assay by measuring the inhibition zones around $NaBF_4$ dopped disks. Zone diameters were obtained for 20 µL of 500 mg/mL $NaBF_4$ solution (Table 3).

Table 3. Agar disk diffusion test results of $NaBF_4$ (mm).

Microorganism	Inhibition zone	PC
E. coli	18	40
P. aeruginosa	16	24
S. typhi	20	36
K. pneumoniae	16	41
A. baumanni	20	10
S. aureus	15	34
MRSA	23	27
C. albicans	16	26
A. niger	14	25
Penicillium sp.	17	25

According to the disk diffusion assay $NaBF_4$ displayed remarkable antimicrobial activity against all microbial species tested. Although there are many studies in the literature that indicate the antimicrobial activities of various boron compounds [31-34], there are few studies that mention the antimicrobial activity of $NaBF_4$. A study conducted by Ghammamy and Keysan (2012) has reported that the synthesized nano-$NaBF_4$ molecules have antibacterial activity against *S. aureus* and Group B *Streptococci* [35]. Another study conducted by Suner et al. (2020) states that protonated polyethyleneimine modified halloysite nanotubes (H-PEI-HNTs) were ion exchanged with $NaBF_4$ ($NaBF_4$-PEI-HNTs) and antimicrobial activity was observed against gram-negative (*E. coli* and *P. aeruginosa*) and gram-positive (*Bacillus subtilis* and *S. aureus*) bacteria, and also a yeast specie (*C. albicans*) [36].

The lowest concentration values of $NaBF_4$ that inhibits the visible growth of the microorganism after overnight incubation (MIC) were determined by the broth microdilution method. The lowest concentration that killed the microorganism was determined by sub-culturing the suspensions onto the agars to look for survivors. Minimum concentration with no microbial growth was evaluated as MBC/MFC. MIC and MBC/MFC results are summarized in Table 4.

Table 4. MIC and MBC/MFC values of $NaBF_4$.

Microorganism	MIC (µg/µL)	MIC (mM)	MBC/MFC (µg/µL)
E. coli	3.906	35.6	3.906
P. aeruginosa	3.906	35.6	3.906
S. typhi	3.906	35.6	3.906
K. pneumoniae	3.906	35.6	3.906
A. baumanni	3.906	35.6	3.906
S. aureus	1.953	17.8	1.953
MRSA	1.953	17.8	15.63
C. albicans	7.813	71.2	7.813
A. niger	7.813	71.2	7.813
Penicillium sp.	7.813	71.2	7.813

MIC results showed that the antifungal and anticandidal efficiency of $NaBF_4$ is lower with respect to its antibacterial efficiency. Moreover, gram-positive bacteria are more susceptible to $NaBF_4$ than gram-negative bacteria. The MBC and MFC values were not different from the MIC values for tested microorganisms except MRSA. MBC was 8 times higher than its MIC value. In literature, there are many studies investigating the MIC values of various boron compounds, however, to the best of author's knowledge there has been no study investigating the MIC values of $NaBF_4$ against any microorganisms. Previously, MIC values of boric acid (BA) and borax were determined by macrodilution method against *S. aureus*, *E. coli*, and *P. aeruginosa* [37]. According to the test results $NaBF_4$ was effective against these microorganisms at 2- and 12-times lower concentrations, compared to BA and borax, respectively. Another study evaluated the MIC values of BA and disodium octaborate tetrahydrate (DOT) against 10 bacterial species. Most susceptible and resistant species are found as *P. aeruginosa* and *S. aureus*, respectively. MIC values of BA and DOT were

determined as 0.385 µg/µL and 0.644 µg/µL against *P. aeruginosa*, 3.09 µg/µL and 10.312 µg/µL against *S. aureus*, respectively [38]. When we compare the MIC results of $NaBF_4$ with DOT and BA, $NaBF_4$ has a higher antibacterial effect than both BA and DOT against *S. aureus*. On the other hand, bactericidal activity of BA and DOT is higher than $NaBF_4$ against *P. aeruginosa*.

The antibacterial activity of $NaBF_4$ for gram-positive bacteria is better than gram-negative bacteria, probably due to their cell wall structure. Cell wall of a gram-positive bacteria is majorly composed of peptidoglycan. On the other hand, a gram-negative bacteria's cell wall is more complex, in addition to the peptidoglycan; polysaccharides, proteins and phospholipids are present in the structure. The charge of the phospholipid is negative, while the peptidoglycan is positively charged [27,39]. When $NaBF_4$ is dissolved in water, it dissociates into Na^+ and BF_4^- ions. Negatively charged BF_4^- ion may interact with positively charged peptidoglycan better than negatively charged phospholipid. As a result of this interaction membrane integrity may be altered and intracellular substances leaked out. This could be the reason why MIC values of $NaBF_4$ for gram-positive bacteria were lower than gram-negative bacteria.

3.2. Cytotoxicity of $NaBF_4$

Cytotoxicity of $NaBF_4$ on L929, mouse fibroblast cell line, was determined by MTT assay and the results are given in Figure 1.

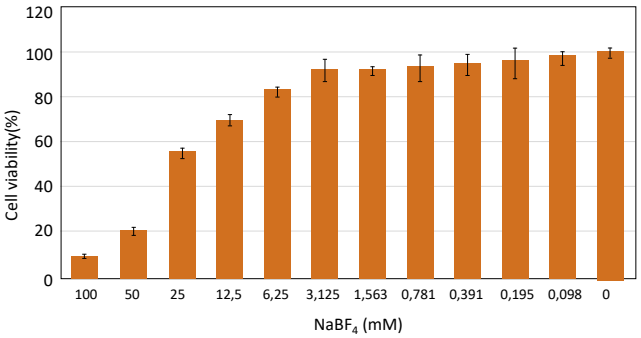

Figure 1. Cytotoxicity of $NaBF_4$ was determined by MTT assay at 24 h culture (n=6).

The cells were cultured in the presence of different concentrations of $NaBF_4$ between 0.098-100 mM for 24 h. The cell viability decreased to 70.26% upon treatment with 12.5 mM $NaBF_4$ and further decreased to 21.25% and 10.33% with 50 mM and 100 mM $NaBF_4$ treatment, respectively. The IC_{50} value of $NaBF_4$ on L929 cells was calculated as 29.3 mM which is a very high concentration. 29.3 mM concentration of $NaBF_4$ corresponds to 3.2 µg/µL concentration. MIC values of *S. aureus* and MRSA with 1.953 µg/µL was lower when compared to the IC_{50} value of L929 cell line. According to these results, MIC values are very close to non-toxic concentration so it can be concluded that $NaBF_4$ can be safely used in tissue engineering studies. In a study, cytotoxicity of $NaBF_4$ was determined with human cervical carcinoma epithelial cells HeLa. The half-maximal effective concentration (EC_{50}) value of $[BF_4]^-$ ion on HeLa cell line was calculated as >25 mM without giving direct concentration [40].

There is a lack of studies investigating the cytotoxicity of $NaBF_4$ in the literature. However, there are some studies investigating the effect of different boron compounds with various cell lines. In a study, IC_{50} value for boric acid on U-87 MG glioblastoma cell line was found as 17 mM, in 48 h culture period [41]. In another study, the combined effect of different concentrations of borax in the presence of 50 µg/mL 5-Fluorouracil (5-FU) on human colorectal cancer cell line, DLD-1 was investigated. 5-FU is a widely used, FDA accepted chemotherapy agent for colorectal cancer. Decreased cell viability and increased early apoptotic cell percentage was shown in the combined application of 5-FU and borax when compared to their application at the same concentrations alone [42]. In a different study, sodium pentaborate pentahydrate (NaB) was used at 15 µg/mL concentration with 10% (v/v) DMSO for freezing. It was concluded that NaB protected viability of A549 cancer cells and L929 cells after 4 freeze-thaw cycles indicating NaB may be a good cryoprotective agent [43].

3.3. Surface Morphology of the Hydrogels

Surface morphologies of the hydrogels were investigated by SEM analysis. Control chitosan surface showed smooth morphology without any roughness. In the $NaBF_4$ modified groups, increased numbers of crystalline particles were found on the surface of the hydrogels with increasing $NaBF_4$ concentration as it is clearly seen from the Figure 2. $NaBF_4$ was dissolved in the chitosan solution before gelation, so this crystallization was triggered during the freeze-drying application. Besides, SEM images obviously shows that $NaBF_4$ crystals were agglomerated during freeze-drying process.

3.4. ATR-FTIR Spectra of the Ch:$NaBF_4$ Hydrogels

The ATR-FTIR spectra of the crosslinked chitosan, $NaBF_4$, and Ch:$NaBF_4$ hydrogels are given in Figure 3. Peaks at 774, 520, 526 and 1332 cm^{-1} are specific for $NaBF_4$ and can be clearly seen in the Ch:$NaBF_4$ hydrogel spectra. These peaks which correspond to characteristic vibrations of $NaBF_4$ [44] were not seen in the spectra of bare chitosan. A broad peak at 3000-3600 cm^{-1}, -OH stretching of the hydroxyl group, was determined for chitosan polymer. The $CONH_2$ and NH_2 groups peaks specific for amide structure of chitosan were recorded at 1651 and 1555 cm^{-1}, respectively [45]. The intensity of the peak at 1555 cm^{-1} was increased with increasing concentrations of $NaBF_4$ indicating the interaction between NH_2 group of chitosan and $NaBF_4$. Sharp peak with broad range at 1055 and 1022 cm^{-1} corresponds to C-O stretching and this peak overlaps with the peak of $NaBF_4$ at 1010 cm^{-1} between the same range.

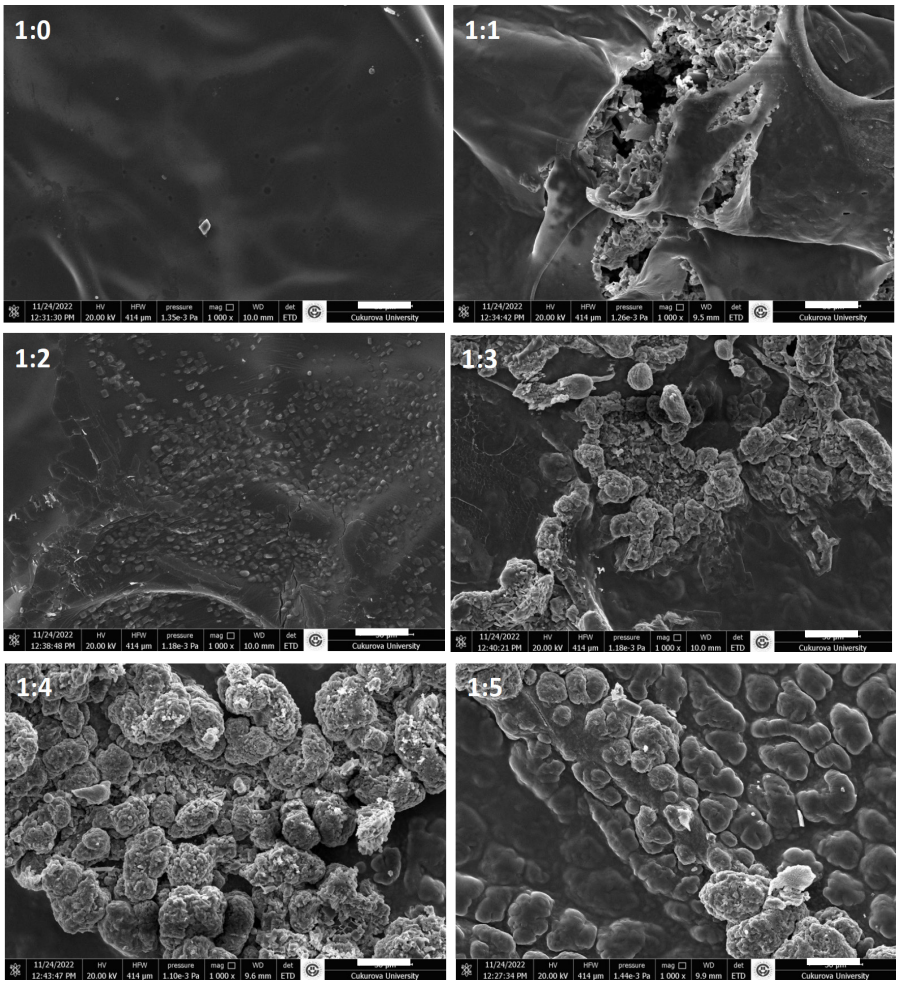

Figure 2. SEM images of Ch:$NaBF_4$ hydrogels with 1000X magnification (Scale bar: 50 µm).

3.5. Agar Disk Diffusion Results of Ch:$NaBF_4$ Hydrogels

Antimicrobial activities of $NaBF_4$ loaded chitosan-based hydrogels were investigated by agar disk diffusion method and, the ratio of the inhibition zone to the sample diameter is given in Table 5, instead of giving the inhibition diameter directly, since each sample could not be obtained at the same diameter. Although chitosan is known as a natural biobased antimicrobial polymer, control groups prepared by using high molecular weight chitosan did not show antimicrobial activity against tested microorganisms. The main reason for the inadequate antimicrobial activity might be related to the molecular weight of chitosan used in this study. Results are coherent with the previous literature studies that support decreasing antimicrobial activity of chitosan with increasing

Table 5. Ratio of inhibition zone diameter to hydrogel diameter.

	Ch:$NaBF_4$					
	1:0	**1:1**	**1:2**	**1:3**	**1:4**	**1:5**
E. coli	N/A*	1.22	2.19	2.22	2.58	2.90
P. aeruginosa	N/A	1.20	3.32	3.82	4.30	4.52
S. typhi	N/A	N/A	N/A	1.85	2.88	3.60
K. pneumoniae	N/A	1.78	2.12	2.19	2.33	2.64
A. baumanni	N/A	N/A	N/A	1.67	1.92	2.16
S. aureus	N/A	N/A	1.36	1.84	2.06	2.26
MRSA	N/A	2.01	2.30	2.44	3.01	3.68
C. albicans	N/A	N/A	N/A	N/A	2.48	3.41
A. niger	N/A	N/A	N/A	1.42	2.04	2.50
Penicillium sp.	N/A	1.60	1.61	2.20	2.51	3.27

*N/A: Not Available.

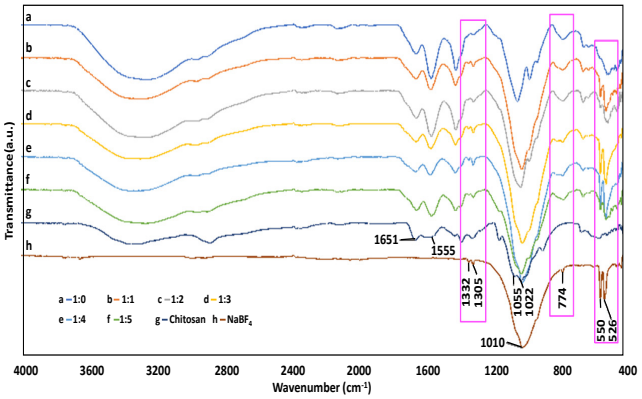

Figure 3. ATR-FTIR spectra of $NaBF_4$ and Ch:$NaBF_4$ hydrogels.

molecular weight [27,46,47]. As described in the previous antimicrobial section, antimicrobial activity could be related to the ionic charge of the biocide itself. Chitosan with positively charged amino groups should interact with the negatively charged cell wall of gram-negative bacteria; however, high molecular weight chitosan have lower pKa and less protonation [27]. This might be the reason why control groups did not show any antimicrobial activity.

Experimental results indicate that inhibition zones increased as the concentration of $NaBF_4$ increased. When we compare the antimicrobial activity among all gram-negative species Ch:$NaBF_4$ hydrogels showed highest antimicrobial activity towards *P. aeruginosa*. Although all gram-negative bacteria, they are different microorganisms from each other, so their susceptibility to any antimicrobial agent may differ from each other. The mode of action of antimicrobial agents such as DNA/RNA inhibition, disturbing enzyme mechanisms etc. may differ according to the type of microorganisms [36]. In a study conducted by Zheng et al. (2003) claimed that increasing molecular weight of chitosan is decreasing the antibacterial activity for gram-negative bacteria while increasing the antibacterial activity for gram-positive bacteria by forming a film which inhibits nutrient adsorption [47]. However, the results obtained from this study could not be associated with the results of the reported study.

Many researchers also used various antimicrobial agents such as metal ions, metal nanoparticles, antibiotics, antifungal drugs, etc. to enhance chitosan's antimicrobial activity [48-51]. In a study by Venkatesan et al. (2014) chitosan-carbon nanotube (CNT) hydrogels were developed by using different CNT concentrations and it was observed that the antimicrobial activity was increased with increasing CNT concentration [46]. In the literature there is no chitosan hydrogel prepared by using $NaBF_4$; however, there are some studies investigating the synergetic effect of boron compounds with chitosan for their potential use as wound care applications. In a study conducted by Huang et al. (2021) chitosan-based hydrogels were prepared by using boric acid or Tris and calcium gluconate containing buffer solutions [52]. According to the results both gels demonstrated tissue repair and anti-infection effects and they were evaluated as potential wound care material for hydrofluoric acid burn therapy. In another study, insufficient antimicrobial activity of chitosan at circumneutral pH of 6.0 was enhanced by using sodium metaborate tetrahydrate and chitosan-borate complexes showed broad spectrum antimicrobial activity against bacteria and fungi [53].

According to the disk diffusion results of this study $NaBF_4$ amount used in 1:3 gels were enough to achieve broad spectrum antimicrobial activity and hydrogels prepared with this formulation can be used as an antimicrobial wound care material (Figure 4).

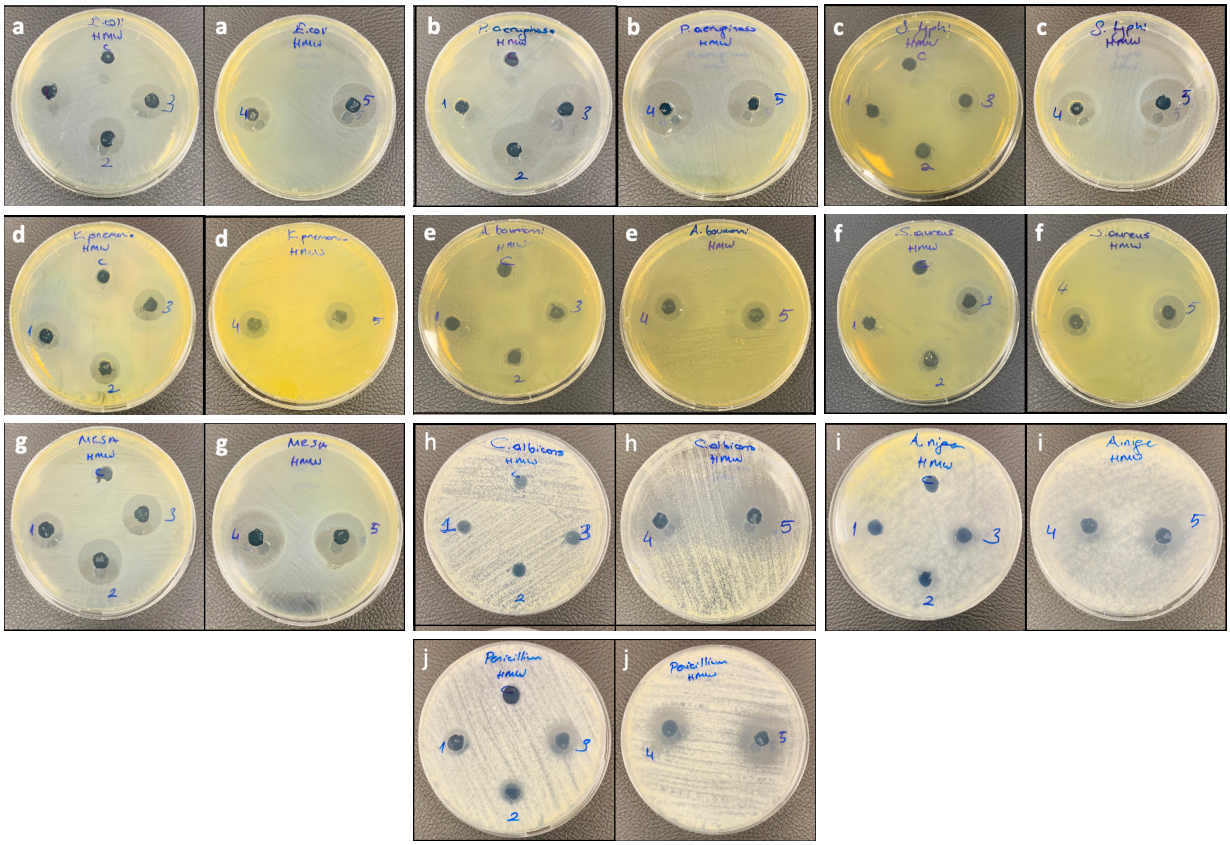

Figure 4. Agar disc diffusion test images of Ch:$NaBF_4$ hydrogels against a. *E. coli*, b. *P. aeruginosa*, c. *S. typhi*, d. *K. pneumoniae*, e. *A. baumanni*, f. *S. aureus*, g. MRSA, h. *C. albicans*, i. *A. niger*, j. *Penicillium* sp.

4. Conclusion

Antimicrobial hydrogels are widely used in wound healing applications due to their numerous advantages. This study is focused on developing antimicrobial chitosan-based hydrogels to be used in topical wound care products. $NaBF_4$ was used as an active agent to enhance the antimicrobial activity of chitosan. The antibacterial properties of $NaBF_4$ were evaluated using *E. coli*, *P. aeruginosa*, *S. typhi*, *K. pneumoniae*, *A. baumanni*, *S. aureus*, MRSA while antifungal properties were evaluated using *C. albicans*, *Penicillium* sp., and *A. niger*. The most effective antimicrobial activity was observed against gram-positive bacteria which are *S. aureus* and MRSA with 1.953 µg/µL MIC value. The IC_{50} value of $NaBF_4$ on the L929 cell line was calculated as 3.2 µg/µL after 24 h culture. Antimicrobial capability of the gels was directly related to the $NaBF_4$ content in hydrogels. Especially, 1:5 ($NaBF_4$ concertation is 910.8 mM) group showed the highest antimicrobial activity against a wide range of microorganisms while the 1:3 ($NaBF_4$ concertation is 546.5 mM) group was sufficient to inhibit microbial growth against all microorganisms used in antimicrobial activity tests. According to the results Ch:$NaBF_4$ hydrogels (1:3, 1:4, 1:5) have the strongest inhibitory effect against *P. aeruginosa* in comparison to the other gram-negative bacteria. Even though the IC_{50} value of $NaBF_4$ on L929 cells was calculated as 29.3 mM which corresponds to 3.2 µg/µL concentration, hydrogels were loaded with higher amount of $NaBF_4$. The reason of this excessive loading is due to the release profile of $NaBF_4$ from the chitosan-based hydrogel which could be discussed in future publication. As a result, the 1:3 sample hydrogel contains a sufficient amount of $NaBF_4$ for preparing an antimicrobial chitosan-based hydrogel which is a promising candidate that can be used as topical wound care product.

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Conflicts of Interest

No conflict of interest was declared by the author.

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