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Interaction of betacoronavirus and *S. aureus* with boron nitride nanoparticles (BNNPs)

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ABSTRACT

Investigations on the advanced effects of boron-containing compounds have gained attention in the last decade. This study was carried out to investigate the effect of hexagonal boron nitride nanoparticles (BNNPs) on Bovine Coronavirus (BCoV) and *Staphylococcus aureus* (*S. aureus*) by different methods. First, the biological effects of different BNNPs concentrations lower than 0.5 mg/mL were examined on HRT-18 (Human Rectal Tumor) for 5 days. Different concentrations of hBN were mixed with BCoV in liquid, on a membrane, or directly on cells and examined for differences in titers or replications. Moreover, Bacterial Filtration Efficiency (BFE) test of hBN powders coated on polypropylene fabric by spray method was applied against *S. aureus*. The compound was found slightly toxic on the HRT-18 cell line by live cell counting, while no remarkable morphological difference was observed. BNNPs treatment with 0.025 or 0.3 mg/mL concentrations did not reduce the infective titer and created no inhibitory effect on in vitro replication. The stability of the virus titer after treatment of BNNPs coated fabric also indicated no antiviral efficiency. But hBN-applied fabric formed a barrier of ≥90.3%, while non hBN-applied fabric formed a≥64.6% barrier for *S. aureus*. The present study demonstrates that BNNPs alone are not a good candidate for disinfectant or drug for BCoVs, while it could be valuable to use as coated fabric in areas needing easy sanitation, especially for *S. aureus*.

1. Introduction

Boron-containing compounds (BCCs) are ubiquitous in nature. It is known that boron, which is mainly used in chemical compounds, is significant in plant nutrition and takes place in the diets of mammals [1,2]. The discovery and widespread research of the biological uses of boron compounds gained attention in the twentieth century. Although the mechanism of action is still mostly not clarified, recent researchers reveal the increasing role of boron in medicinal chemistry and drug design. BCCs have broad application areas in preventative (as an antiseptic and vector disease control), diagnostic, and therapeutic applications [3]. BCCs can be used as biomolecules in boron neutron capture therapy (BNCT) and in detecting biological markers of diseases [1,4]. The high adsorption rate of boron increases its use in drug development, diagnosis, and treatment [3,5]. As an antibiotic and antiseptic, boron has also other therapeutic properties such as anti-fungal, anti-coagulant, anti-diabetic, anti-hypertensive, anti-nociceptive, anti-parasitic and antiviral [1,3-7].

On the other hand, nanotechnology has also gained potential in biomedical applications. Nanoparticles (NPs) are complex molecules used for clinical diagnosis, treatment of diseases, and biomedical imagining, known as nanomedicine [8]. While copper and silver NPs demonstrated as toxic to microorganisms, boron has been suggested as functional in host defense [8].

Boron nitride (BN) is one of those boron-containing nanomaterials which has multiple purpose features. BN has different polymorphisms as; cubic BN (cBN), hexagonal BN (hBN), wurtzite BN and rhombohedral

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BN (rBN) [9]. Among them, hBN has potential and several successes in drug carrier systems, increasing drug or biomolecule delivery, gene delivery, biosensors, and tissue engineering [9]. There are many studies on the use of hBN in the form of nanotubes, nanosheets, nanocages, and nanoparticles. While BNNPs have many layers, nanosheets have a few layers. Typically, lengths in one of the three dimensions of nanoparticles have a size between 1 and 100 nm, it plays a primary role in their longer circulation half-life, biodistribution, and clearance [10]. Recent studies have shown that the antibacterial activity of BN nanosheets doped with zirconium (0.5, 1.0 mg/50μL) enhanced against *Escherichia coli* (*E. coli*) and (*Staphylococcus aureus*) *S. aureus* [11]. Additionally, hBNNPs have been indicated high biofilm activity and inhibiting bacterial growth for *Staphylococcus pasteuri* (*S. pasteur*i) M3, *Staphylococcus mutans* (*S. mutans*) ATTC 25175, S. mutans 3.3, and Candida sp. M25 agents [12]. The antimicrobial activity of BN nano flake-polymer composite samples has been investigated against *E. coli, Pseudomonas aeruginosa, Staphylococcus epidermidis* (*S. epidermidis*), and *S. aureus*. The results have indicated that BN nano flakes physically interact with the bacterial cellular envelope, leading to irreparable physical damage, and also BN can minimize infections that may be associated with biomedical devices [13].

Due to the impact of the SARS-CoV2 pandemic raised in 2020, the activities of nanomaterials on viruses have become more important. The effects of BNNPs on viruses have not been studied yet. In this study, it was aimed to investigate the efficacy of hexagonal boron nitride nanoparticles on Bovine Coronavirus (BCoV), as a model of betacoronaviruses. Also, their biological compatibility on the Human rectal tumor (HRT-18) cell line and bacterial filtration efficiency for determination of bacterial permeability were studied.

2. Materials and Methods

2.1. Materials

Polypropylene (PP) non-woven fabric has an average of 100 g/m^2 and 0.55 mm thickness, provided from Mogul Company (Gaziantep, Türkiye). hBN powder (99.97% purity) was supplied from BORTEK Inc. (Eskişehir, Türkiye). Trimethyl methoxysilane (TMS) used as the bonding and adherent agent were purchased from Sigma Aldrich. ORGAL 430, the acrylic resin used as a binder, was obtained from Organic Chemicals Ltd. Sti. All chemicals used are of analytical purity.

2.2. Methods

2.2.1. Preparation of BNNPs and BNNPs coated fabrics

The dispersed BNNPs were obtained through continually stirring for 24 h in distilled water and after that sonication process (SONICS 750) for an hour. After the dispersion process (3.0 wt.%), a certain amount of trimethyl methoxysilane (2 vol%) dissolved in a suspension by stirring. The mixture was stirred at room temperature for 2 h, followed by adding a binder (1g/100mL). The prepared solution was filled into pressurized spray cans and applied to fabrics. Thermal treatment was applied at 110°C for 2 h to form covalent bonds between the silane coupler and nanosheets besides fixation to the fabric.

2.2.2. Characterizations

The density of hBN was calculated using the Helium pycnometer (Quanthachrome Multipycnometer). The grain size distribution was determined using the Malvern Mastersizer Hydro G2000 grain size measuring device. Fourier transforms infrared (FT-IR) spectroscopy in the attenuated total reflectance (ATR) mode (Bruker, Tensor 27) was utilized to measure whether raw materials, fabric, and silane grafted BNNSs were successfully coated onto the fabric surface. The measurements were done in a spectral range from 700 to 4000 cm^{-1} with a resolution of 4 cm^{-1} and 32 scans. The data were collected and processed using OPUS Software. The microstructure and morphology of BNNPs, silane grafted BNNs, and coated fabrics were identified by X-Ray diffraction patterns (XRD, Rigaku, Miniflex 600, Japan, Cu Kα radiation, λ=0.15406 nm) and scanning electron microscope images (SEM, ZEISS Supra 50VP). Before observation, the samples were coated with gold/palladium using a sputtering coater.

2.2.3. Virus, bacteria and cell line

BCoV, Mebus strain was used for testing the antiviral activities of hBN. While the virus used had an early passage (5p) level, the infective titer (50% tissue culture infectivity dose, $TCID_{50}$) for the test virus was $10^{6,25}$. The virus was obtained from our stock of laboratory. Human Rectal Tumor (HRT-18) cell line was used to propagate and titrate the viruses. Cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with heat-treated 10% fetal calf serum (FCS), 100 UI/mL Penicillin/Streptomycin, and 250 μL/mL Amphotericin B solution. Cells were incubated at 37°C, in a 5% CO_2 atmosphere. *S. aureus* ATCC 6538 was used for the bacterial filtration efficiency test. The bacterium was enabled from Biotechnology Laboratory, Eskişehir Osmangazi University, and was grown in a Brain Heart Infusion medium. *S. aureus* was grown up at optimum at 37°C.

2.2.4. Preparation of BNNPs for experiments

The BNNPs were dispersed in PBS to be adjusted as 1mg/mL. The pH was not balanced during preparation. When the measurement was made, it was determined that the pH of the prepared solution was 7.0. To eliminate the negative effects that may occur in cell cultures, the compound was autoclaved at 121°C for 15 minutes. After autoclaving, the solution was rotated

on a magnetic stirrer for 24 h to obtain a homogeneous mixture. Just before the experiments, this mixing process was carried out at 100°C for 10 minutes. Then, the solution was used in the experiments when it was cooled-down to 37°C.

2.2.5. Cell viability assay

HRT-18 cells were prepared at a concentration of 100,000 cells/mL in 24-well plates one day before the assays. For cell viability assay, the culture fluids were collected from morphologically healthy cells with fully covered wells. Seven concentrations (0.5, 0.4, 0.3, 0.2, 0.1, 0.05, 0.025 mg/mL) were prepared by diluting the BNNPs dispersed with sterile PBS and were added to the allocated wells for each dose. The well plates were incubated at 37°C with a 5% CO_2 atmosphere for 5 days and were examined daily by inverted light microscopy. During the test period, cells from individual wells were collected by trypsinization and centrifuged at 1500 rpm for 10 min. Cells were stained with trypan blue after the cell pellet was diluted with 1mL of DMEM. The viability rate, calculated as the percentage of living cells to total cells, was recorded with 24 h intervals.

2.2.6. Experimental application on BCoV

2.2.6.1. Effects of BNNPs on free virus particles in a liquid environment

For this experiment, equal volumes of BNNPs at different concentrations and 100 $TCID_{50}$ BCoV diluted in DMEM were mixed in a glass tube at room temperature. Also, an equal volume of 100 $TCID_{50}$ viruses and PBS were mixed for BNNPs-free conditions as negative (blanc) control. This experimental design was applied for two concentrations (0.025, 0.3 mg/mL hBN) selected according to predetermined cell viability analyses. However, this experiment was also carried out with a little modification for a higher concentration whose effect on the cell was not determined (1mg/mL). The BNNPs/virus mixtures prepared in glass tubes were incubated at room temperature 23±1°C for 0, 1, 2, and 6 h. During this period, the mixture was homogenized using a vortex every hour, even before sampling. At each represented hour, 100 µL of suspension was taken and used for the titration assays.

The mixture prepared in high concentration (1mg/mL) and its negative-control tube, were incubated at room temperature for 1 h. In order to exclude the adverse effect of the precipitated excessive amount of BNNPs during the microscopic examination, suspensions were centrifuged at 1500rpm before sampling. Then, 100 µL of suspension was used for the titration assays. The experimental setup was repeated twice for each condition.

2.2.6.2. Effect of BNNPs on virus replication

For analyzing the antiviral activity of BNNPs on infected cells, 24-well plates were used. Wells coated with 100,000 cells/mL were prepared 24 h before the assay. The next day, when the cells covered almost the entire well, cell culture fluids were removed and cells were infected with 100 $TCID_{50}$ virus suspensions. The plates were incubated for an hour at 37°C for virus adsorption. The inoculum was removed and monolayers were washed with PBS. Serum-free medium containing different pre-determined concentrations of BNNPs (0.025, 0.3 mg/mL) was added to the cells. Cells were daily examined for morphological changes for 5 days. And also, titration assays were conducted to collect samples from the relevant wells each day during this period. The experimental setup was repeated twice.

2.2.6.3. Effect of BNNPs on serial virus passages

In this test design, the collected samples from the previous experiment were used to identify the BNNPs effect on the sensitivity of viral replication in recurrent infections. For this purpose, 24-well plates were coated with HRT-18 cells with a concentration of 100,000 cells/mL. Cells culture media was removed and 200 µL of each sample was inoculated on a different well without dilution. The plates were incubated for an hour at 37°C and the virus growth medium (DMEM without FCS) was added. In this experiment, only a microscopic examination was carried out. And it was investigated whether there were cytopathogenic changes (CPE) due to the virus replication.

2.2.6.4. Exposure of BCoV on BNNPs coated fabric

BNNPs coated fabrics were cut in 2*2 cm^2 sizes and placed in petri dishes were sterilized by autoclaving at 121°C for 15 minutes prior to use. Three dishes were used for experimental conditions.

- *Petri dish A:* 100 $TCID_{50}$ BCoV with a volume of 200 µL was placed on the BNNPs coated fabric and incubated at room temperature. Consecutive samples were taken at the 2^{nd}, 8^{th} and 24^{th} hours after adding the virus suspension to the fabric.

- *Petri dish B:* 100 $TCID_{50}$ viruses with a volume of 200 µL was added to the BNNPs -free fabric and incubated at room temperature. The sample was taken only at the 8^{th} hour after adding the virus suspension.

- *Petri dish C:* 100 $TCID_{50}$ virus with a volume of 200 µL was added to an empty petri dish as control and similar conditions are provided.

Each of the fabrics was collected from dishes at designated times and placed in falcon tubes. After adding 3.8 mL of DMEM to it, it was vortexed for 5 minutes, allowing the viral particles on the fabrics to pass into the liquid phase. This obtained virus suspension was filtered using 0.2 nm PES filters (ISOLAB, Türkiye) and then titrated for the number of live virus particles.

2.2.6.5. Virus titration assay

To measure the antiviral activity of BNNPs, the $TCID_{50}$ was determined for each concentration for every sampling period of the experiments. For this purpose, serial dilutions (10-fold) of samples were made in DMEM. For each sampling 4 replicate wells of 96-well tissue culture plate were used. The virus diluent of 50 µL and an equal amount of cell suspension (2×10^5 cells/mL) was added to all the test wells and incubated at 37°C, 5% CO_2 atmosphere. Cells were observed daily by an inverted light microscope and the $TCID_{50}$ per milliliter was calculated according to the Spearman-Kârber method.

2.2.7. Bacterial filtration efficiency experiment

The Bacterial Filtration Efficiency (BFE) test of hBN powders coated on polypropylene fabric by spray method was applied according to standards including ISO11737-1 and TSE-EN 14683+AC. It has been made compatible with the requirements of ASTM (American Society of Testing and Materials) F2101. This test was performed in a sterile cabin under aseptic conditions. PP blank and PP-hBN fabrics were measured using *S. aureus* ATCC 6538 in airflow of 14 L/min. The bacterium suspension was applied as an aerosol by a nebulizer on the outside of two materials. The concentration of *S. aureu*s was diluted as 1700-2700 cfu/ml (colony-forming unit) mentioned in the standard procedure. BHI (Brain Heart Infusion) agar and normal saline (NaCl, 0,9%) were used for the growing bacteria and dilutions, respectively. The aerosol sample was sprayed from one side (from the outside of the mask), and the bacteria passing through the other side (to the inside of the mask) were collected onto a petri dish. Planted petri dishes were incubated at 37ºC overnight then the growing colonies were counted. The mean values obtained in duplicate experiments were used to calculate the BFE [14,15].

Positive control was a bacteria suspension that was sprayed directly without materials onto a petri dish. After the incubation, the colonies were counted. Equation 1 was used for the calculation of BFE %. 1% was accepted as low limit detection, and 99% was accepted as high limit detection [15].

$$BFE\ (\%) = \frac{Positive\ control\ (CFU) - Test\ Sample\ (CFU)}{Positive\ control\ (CFU)} x100 \qquad (1)$$

3. Results

3.1. Characterization of Hexagonal Boron Nitride

In a previous study, the characterization of commercial hBN powder was done and similar results were obtained [16]. Briefly, it was determined that the density of hBN powder used in experimental studies was 2.01 g/cm^3 and the tap density of the powder was in the range of 0.4-0.5 g/cm^3. The specific surface area of the hBN powder was measured as 22 m^2/g. The average particle size distribution of hBN was measured and D_{10}, D_{50}, and D_{90} values were 0.050 µm, 0.098 µm, and 0.210 µm, respectively. The mineralogical analysis of hBN was determined by the x-ray method (Figure 1a) and the sharp peaks in the diffraction pattern are characteristic peaks of hBN with lattice parameters a=2.57Å, c=6.70Å (JCPDS No: 034-0421). It was determined that hBN was well crystallized and free of impurities.

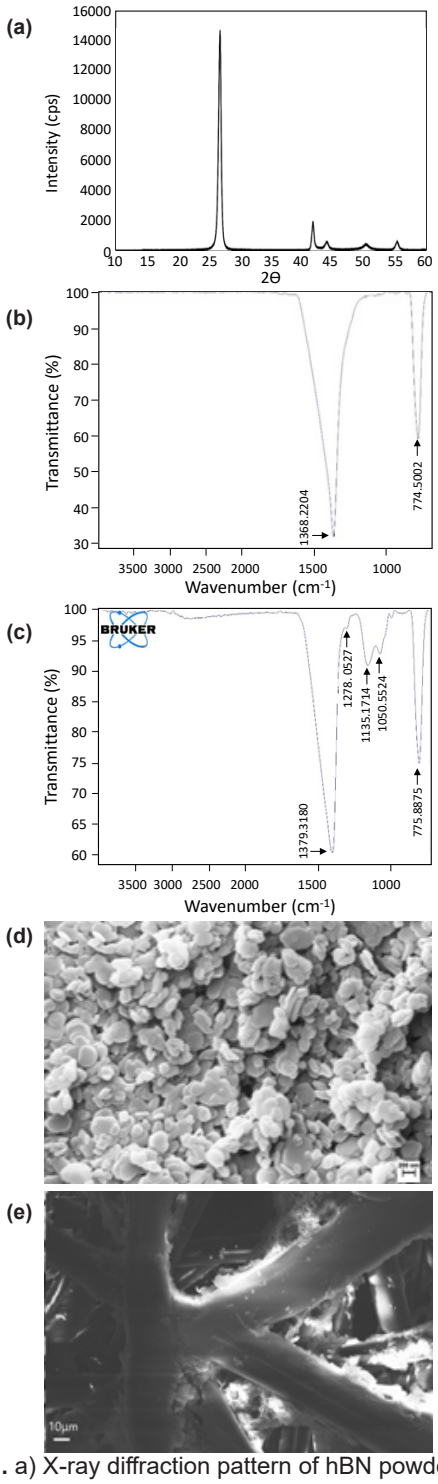

Figure 1. a) X-ray diffraction pattern of hBN powder, FTIR spectrum of b) hBN powder and c) silane modified BNNPs. SEM images of d) hBN nanoparticles and e) Fabric after silane decorated BNNPs coating.

FTIR technique allows the chemical composition and bonding arrangement of the components in the powder to be determined. To verify the purity of the BN powder, FTIR analysis was performed (Figure 1b). The bands detected at 774.5 cm^{-1} with 1368 cm^{-1} belong to the hexagonal phase of BN. While the peak at 774.5 cm^{-1} corresponds to B-N-B vibrations, the band at 1368 cm^{-1} was caused by stretching vibrations of B-N. No other bands were observed, which means that there is no bonding on the particle surface. Its morphological structure is shown in Figure 1d, e. The SEM image revealed that BN particles had diameter of 50-200 nm and thickness of 50-75 nm with a rounded shape.

3.2. Characterization of BNNPs Coated Fabric

Since silane coupling agent TMS have unique chemical composition, they act as binders between BNNPs and PP fabric. Figure 1c also shows the FTIR analysis of the suspension after drying at 100°C. While 1379 cm^{-1} and 775.8 cm^{-1} bands show hBN characteristic binding, the bands located at 1278.05, 1135.17, 1050.5, and 970 cm^{-1} in the fingerprint region show that the silane agent interacts with BN. The reason for BN characteristic peaks shifting may be the introduction of chemical groups of silane agents in the BN network. 1278 cm^{-1} is possibly from C-N stretching of amines that of 1050 cm^{-1} may be due to asymmetric stretching of Si-O-C [17]. There are peaks found at 1130 cm^{-1} and 970 cm^{-1} that could be assigned to Si-O-Si [17,18].

Silane modified BNNPs coated and uncoated PP fabrics were examined by FTIR to investigate what chemical interaction happens at the surface of the polymer (Figure 2). All dominant bands observed in PP fabrics (red line) were characteristic bands showing the isotactic structure of PP. Asymmetric stretching vibrations of CH_3 and CH_2 groups were observed in the spectrum corresponding to wavenumbers of 2958 and 2919 cm^{-1}, respectively. While symmetric stretching of CH_3 (methyl group) was observed at 2875 cm^{-1}, symmetrical and asymmetrical scissoring vibrations were observed at 1458 and 1375 cm^{-1} wavenumbers, respectively [19,20]. As 1376 cm^{-1} and 777 cm^{-1} bands show hBN characteristic bonding, the vibration bands located at 1278.05 and 1135.17 in the fingerprint region supports that the silane agent formed a bond with BN. A new absorption band was found at 1731 cm^{-1} in the treated fabric. It was probably caused by C=O stretching vibration. According to FTIR analysis, silane decorated BNNPs successfully formed a barrier film on PP fabric.

The surface morphology of the polypropylene fabric after silan decorated BNNPs coating is shown in Figure 1d, e. It can be said that the coating thickness is not homogeneous depending on the spray application. BNNPs were distributed on PP fibers and at fiber intersections, and BNNPs were also found to penetrate into the PP fabric.

3.3. Cytocompatibility of BNNPs to HRT-18 Cells

Biocompatibility of BNNPs in *in-vitro* cultures, determined by treating HRT-18 cell line with seven different concentrations (0.5, 0.4, 0.3, 0.2, 0.1, 0.05, 0.025 mg/mL) of the BNNPs. Cell viability rate was analyzed over 5 days. All concentrations remained above 50% viability during the evaluation period (Figure 3). In all the data obtained during 5 days, it was determined that death cell rates varied between 0.2% and 38%. On the fifth day of the treatment, viable cell count rates for concentrations from 0.5% to 0.025% were as follows; 91.89, 89.65, 93.5, 89, 84.4, 84.6, 90.4% for treatments and 94.4% for non-treated cells, respectively. It was observed that the maximum cell deaths were on the 2nd day for most concentrations and decreased in the following days.

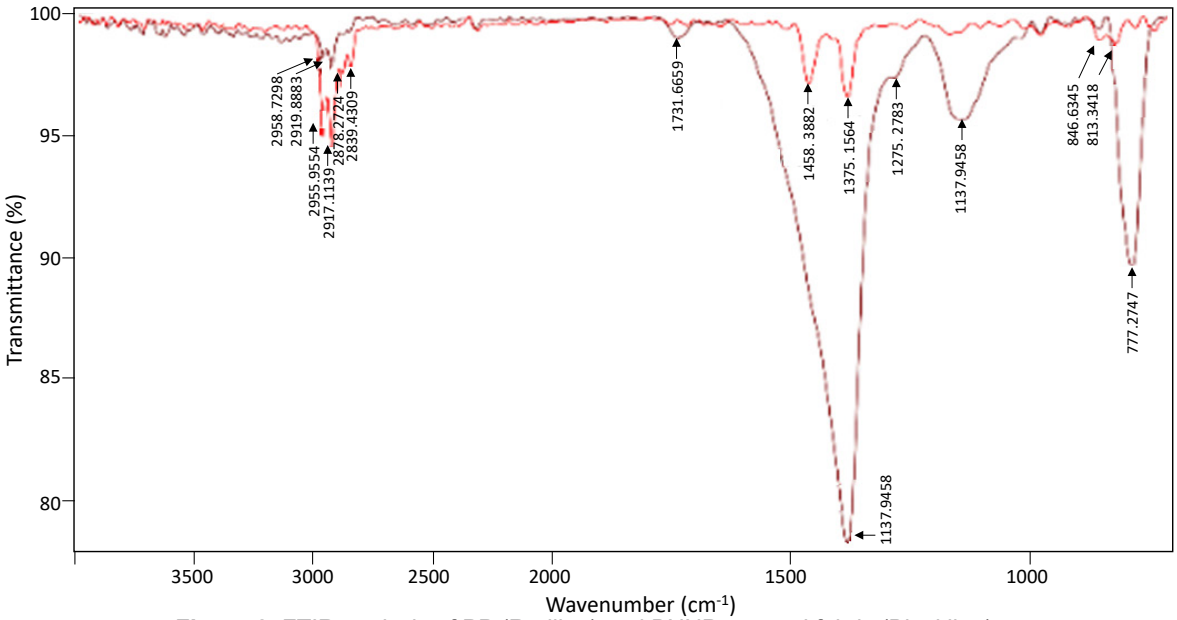

Figure 2. FTIR analysis of PP (Redline) and BNNPs coated fabric (Blackline).

Also, it was determined that the BNNPs in the medium did not have a negative effect on the cells in following days, and even the number of cells increased at all concentrations. In the evaluation of the 5th day, it was observed that the rate of cell death varied between 0.9-10 percent

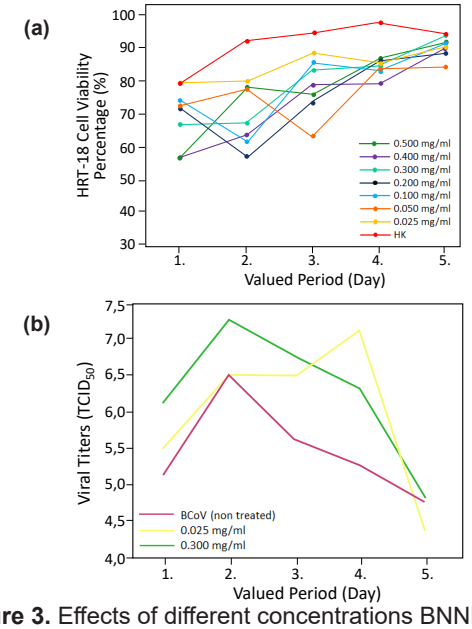

Figure 3. Effects of different concentrations BNNPs on cell viability and BCoV infectivity.

3.4. In Vitro Microscopic Analyses

Apart from cell viability rates, cells were further examined for morphological changes under an inverted microscope on all the days of the sampling period designed for the cell viability assay. No toxic effects or morphological differences were observed at the evaluated concentrations. However, it was observed that the BNNPs accumulated extensively at the cell-cell boundaries at concentrations of ≥0.2 mg/mL. Especially at the concentrations of 0.4 and 0.5 mg/mL, accumulation of the product was at a level that might be made it difficult to microscopically examine the CPE development led by virus growth (Figure 4). For this reason, in order to accurately evaluate the morphological changes in virus infected cultures, it was decided to study with concentrations of 0.3 mg/mL and below in cell-related experiments.

3.5. The Effect of BNNPs on Free Virus Particles in a Liquid Environment

In this experimental design, the inactivation activity of BNNPs was examined by combining both BNNPs and BCoV particles in the liquid phase and keeping them at room temperature for whether there was a difference in virus titer by time. Virus titer values of samples taken at 1, 2, and 6 hours for 0.025 and 0.3 mg/mL concentrations compared with values from untreated BCoV determined not even a half-log difference at either concentration or across all sampling periods (Table 1). On the other hand, when values of 1 mg/mL concentration were compared with BNNPs-free virus suspension, there was also no significant difference at all time intervals as well.

Table 1. Titer values at different time intervals of boron-treated BCoV in liquid phase.

Sampling hours	Viral titers (Log10 $TCID_{50}$)		
	BNNPs-free BCoV	0,025 mg BNNPs	0,3 mg BNNPs
0.	$10^{1,75}$	$10^{1,75}$	$10^{1,5}$
1.	$10^{1,75}$	$10^{2,0}$	$10^{1,75}$
2.	$10^{1,75}$	$10^{1,75}$	$10^{1,5}$
6.	$10^{0,75}$	$10^{1,75}$	$10^{1,5}$

3.6. The Effect of BNNPs on Virus Replication

Effect of BNNPs on BCoV replication was examined by adding BNNPs containing medium after virus

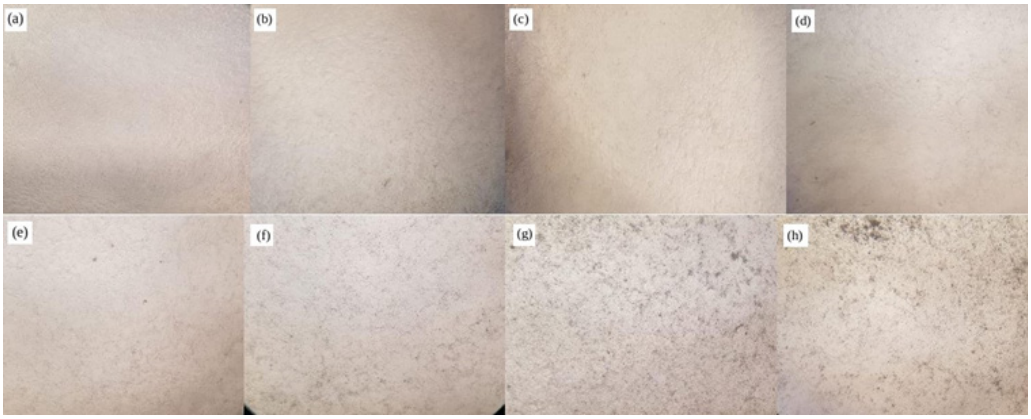

Figure 4. Microscopic images obtained from cell viability assay on the 3rd day treated with different concentrations of BNNPs (4x magnification): a) BNNPs free cell, b) 0.025 mg/mL treated cells, c) 0.05 mg/mL treated cells, d) 0.1 mg/mL treated cells, e) 0.2 mg/mL treated cells, f) 0.3 mg/mL treated cells, g) 0.4 mg/mL treated cells, h) 0.5 mg/mL treated cells. Accumulation on the cell which limits differentiation of slightly occurred CPE effects in the culture.

attachment. According to the mean values of the test results, both concentrations (0.025, 0.3 mg/mL) did not inhibit the virus replication until the 4th day of the experiment, even an enhancer effect on virus replication has been determined. When the titer values of BNNPs non-treated virus were compared with the titer values of virus grown in the presence of both concentrations (0.025, 0.3 mg/mL) of BNNPs, it was seen that the increase both in the 3rd day and 4th day values were higher than one log (Figure 3). It was observed that the mentioned effect disappeared on the 5th day. Virus titers (log 10) of BNNPs free, 0.025, and 0.3 mg/mL treated virus suspensions were evaluated as 4.75, 4.4, and 4.8, respectively. The obtained results were very close to each other, though shows that the treatments are not effective on BCoV replication. Although the experimental setup was carried out in 2 repetitions, after the results were obtained, the titration process was repeated for confirmation, and similar results were obtained.

3.7. The Effect of BNNPs on Serial Virus Passages

Samples obtained from both BNNPs-treated virus suspensions (0.025 and 0.3 mg/mL) and BNNPs free virus suspension as a control in the previous experiment were further passaged. This process was repeated twice. In both replicates, it was observed that effective virus replication could be yielded not only in the BNNPs-free, but also BNNPs treated experiment at both 0.025 and 0.3 mg/mL concentrations.

3.8. The Effect of Membrane Coated BNNPs Against BCoV

To determine whether BNNPs-containing compound inhibited the infectivity of BCoV particles when they came together on a fabric, infective virus particles were perfused on a BNNPs coated fabric and treated for 24 hours. The data obtained from this combination were compared with the infectivity of both pure virus and virus particles suspended on the uncoated fabrics. Results of BCoV (pure control virus) were detected as $10^{5.75}$ while BCoV titers collected over; membrane free of BNNP at 8th hour, membrane coated with BNNPs at 2th, 8th and 24th hour were all $10^{4.25}$. More than 1 log (Log 10) decrease between the titers of pure BCoV and the virus collected from the uncoated fabric. However, the titer of virus suspensions treated with the BNNPs-coated and uncoated fabrics were at the same level after incubation for 2, 8, and 24 hours.

3.9. Bacterial Filtration Efficiency Analyses

S. aureus aerosol was only applied on the coated surface. According to the using direction of the fabric, bacteria permeability from the outside to the inside has been tested. BFE % was calculated by using Eq.1. As a result of the tests, it has been determined that the PP blank sample creates a barrier of ≥64.6% and the PP-hBN sample a barrier of ≥90.3% in the application made from the outside to the inside. It can be said that the hBN applied to the fabric surface protects by reducing bacterial permeability.

4. Discussion

According to recent findings, boron containing compounds have low toxicity for humans and animals similar to table salt [1]. BNNPs are a unique boron compound that have recently attracted attention in medical practice. But their use as a medical material is related to their properties such as size, shape, form, and surface area [9].

Despite the fact that only limited reports are available on the effects of BNNPs to date, there are various studies describing the antiviral effects of BCCs [21-30]. For instance, proteasomal inhibitor, 0.1 μM Bortezomib was found to inhibit Influenza A (IAV) virus at over 100-fold (2 fold at Log 10). However, the compound was found to be highly toxic on MDCK cells and expressed to have no practical utility to inhibit IAV replication in this in vitro system [31]. Horváth et al. evaluated that a concentration of 2 μg/mL BNNT was cytotoxic for all the cell types (lung alveoli and human embryonic kidney cells) studied for 5 days [32]. In another study, 0.4 μg/mL BNNPs revealed low cytotoxicity in MDCK cells but a significant reduction with the same concentration in normal human skin fibroblast cells on the second day [12]. Therefore, it is possible to mention that the toxicity values for BCCs vary in different cell lines. Differences were associated with physical forms, coatings, dispersion procedures of BNNP, preferred cell types, and toxicological assays in *in-vitro* studies Kıvanç et al. showed that BNNPs nanoparticles could be considered as a potentially safe oral care product up to 0.1 mg/mL concentration [12].

Comparing the previous investigations this study presents a longer evaluation (5 days) for toxicity by exposing HRT-18 cells to low concentrations of BNNPs. As <20% reduction in the number of live cells referred to as slight cytotoxic in a previous study [12], in the present study, we determined all the tested concentrations as slightly toxic on the HRT-18 cell line and no remarkable difference in cell morphology was observed. But BNNPs accumulation observed on cell monolayer can create a negative effect on the visuality of cell morphology or may be deterring on *in-vitro* studies performed for determining the virus replications by microscopic analyses of the cell cultures.

Nevertheless, it was preferred to study concentrations of BNNPs, where 0.025% was the lowest concentration tested in cytotoxicity assay and also 0.3% was the highest concentration where the relatively low level of BNNPs accumulation on cells was determined by microscopic evaluations. However, according to the results obtained from viable cell numbers for both of the selected concentrations, the cumulative accumulation did not lead to cell death, and the proliferation rate seen in BNNPs-treated cells was similar to non-treated ones.

A dipeptide-boronic acid analog (Bortezomib/PS-341), has a proteasome inhibitor nature, and had shown a significant reduction of progeny *Severe fever with thrombocytopenia syndrome virus* (SFTSV) titers in infected cells [30]. Virus replication inhibition for *Hepatitis B virus* as long as 6 days with a single intravenous dose of 1 mg of bortezomib/kg was also accomplished *in vivo* [29]. The protease inhibitor effectiveness of BCCs has also expressed for *Vesicular stomatitis virus, Venezuelan equine enceph* [21-28].

Recently, Cetiner et al., demonstrated the possible suitability of BCCs as an antiviral agent against SARS-CoV-2, by molecular docking [33]. A BCCs binding site in the M^{pro} region of SARS-CoV-2 was also reported [34]. Moreover, it is clear to mention the necessity for confirmation of the molecular docking studies by in vitro testing of the molecule-virus interaction. Despite there is no previous molecular docking study on compatibility between BNNPs and BCoV, we performed a direct treatment of BNNPs to BCoV suspension, which resulted in no significant effect on the BCoV titer, suggesting there is no direct antiviral effect of BNNPs. These data show that the possibility to use of BNNPs as a disinfectant is limited.

Interaction of BCCs with drug molecules makes the therapeutic agents more favorable and efficient [35]. At a treatment study, the combination of bortezomib and acyclovir, an anti-herpes drug, inhibited *Herpes Simplex* infection more effectively comparing treatment with drug alone, and also had reduced the infectivity of acyclovir resistant HSV-1 strains [36]. In the present study, BNNPs are tested alone (without interaction with an antiviral drug) and no inhibitory effect on BCoV replication presented by viral infectivity assay was demonstrated. This result represents that BNNPs do not limit virus growth in vitro and are not successful drug candidates alone. But, in analogy to previous studies [36], it is an open area to test, in the case of combined use, the effect of BNNPs on the efficiency of antiviral drugs which already exist in the market.

Coating the material surfaces and fabrics by an antiviral preparation could serve as an efficient approach for preventing from some contact infections [37]. The efficiency of BNNPs as well as other BCCs as a fabric coating material in various fields has already been reported [38,39].

In the present study, hexagonal boron nitride nanoparticles were investigated for inhibition of BCoV with direct contact either on nanoparticle coated fabric or in liquid form. The BNNPs concentrations of 0.3 and 0.025 mg/mL did not make changes in the virus titers in liquid phase analyses. Despite the concentration of 1 mg/mL BNNPs was not evaluated in cell viability test, it was included in the analyzing the inhibitory effect by direct treatment. Similarly, no significant reduction in virus titer was observed by using 1 mg/mL BNNPs in liquid form. No inhibitor effect of those of tested concentrations on BCoV replication was observed either.

With the Covid-19 pandemic, the research on face mask materials has increased. There are many studies conducted with different mask materials including PP which is the most preferred [40,41]. PP is one of the most preferred materials for mask manufacturing because of its rare allergen characteristics [42]. Studies on the mask materials like cotton, polyester, nylon, and silk have shown around 5-25% bacteria filtration efficiencies [40]. Medical masks manufactured with different techniques including non-woven spun-bonded and Meltblown have shown >99 % BFE [43]. The mask materials associated with spun-bonded PP showed low filtration efficiency [41,43]. The masks composed using one layer or less than three layers of polypropylene spun bond have better breathability than others, but the BFE of these masks has remained below the threshold set by the standards [43]. Therefore, masks manufactured with a combination of different techniques including non-woven spun-bonded and Meltblown have shown >99 % BFE [43]. The coated materials have been studied and shown a positive effect on BFE %. While the BFE of PP fabrics coated with mangosteen extract was measured as >95% [44], the BFE of PP coated with alginate copper (II) was not reported as the characteristics of viral inactivation were reported [45]. Although no antiviral effect was found in this article, it was evaluated that BNNP can be used successfully in face masks, especially because it increases bacterial filtration efficiency.

5. Conclusions

Advances in boron chemistry expanded the field of research and the use of this element in medical chemistry. However, there is an insufficient number of studies on hBNNPs, especially on viruses, among the extensive studies of boron-containing compounds. The present study demonstrates that low dose BNNPs alone is not a good candidate to use as a disinfectant or drug on BCoV but coated materials have been studied shown a positive effect on *S. aureus*. However, it could be valuable to use as coated fabric in areas needing easy sanitation. The non-cytotoxic effect obtained in this study indicates that research evaluating the role of BNNPs as a carrier rather than the antiviral activity would be favorable in future work.

Conflict of interest

The authors declare that they have no conflicts of interest.

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